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(54) Title: NEUROPROTECTIVE, ANTITHROMBOTIC AND ANTI-INFLAMMATORY USES OF ACTIVATED PROTEIN C (APC)

(57) Abstract: The present invention provides methods for treating subjects having or at risk of having a neuropathological disorder or brain inflammatory diseases with and without vascular involvement, and systemic inflammatory vascular disease by administering a therapeutically effective amount of Activated Protein C (APC) to the subject. Brain disorders and brain inflammatory vascular diseases that can be treated by the invention method include all neurodegenerative diseases with different types of neuronal dysfunction, including stroke, Alzheimer's disease, Parkinson's disease, Huntington disease, neuroimmunological disorders such as multiple sclerosis and Gullian-Barre, encephalitis, meningitis, as well as other peripheral vascular diseases, such as diabetes, hypertension, arteriosclerosis. Also included are methods of treatment using APC in combination with a co-factor, such as Protein S.

NEUROPROTECTIVE, ANTITHROMBOTIC AND ANTI-INFLAMMATORY USES OF ACTIVATED PROTEIN C (APC)

FIELD OF THE INVENTION

The present invention relates generally to methods for using a new class of neuroprotective agents with additional anticoagulant, anti-inflammatory effects for treatment of pathologies, and more particularly to uses of activated protein C (APC) for the treatment of such pathologies.

BACKGROUND OF THE INVENTION

Serine proteases are a class of proteins that proteolytically cleave other proteins. Members of this class of proteins contribute to important biological processes including the proteolytic cascade reactions of complement activation and blood coagulation. Cleavage of a blood coagulation factor contributes to the coagulation cascade, resulting in blood coagulation. A variety of medical conditions can arise where it is advantageous to inhibit the coagulation cascade at the level of one or another proteolytic step. In addition, procedures involving blood product manipulation can activate members of the cascade, and therefore their specific inhibition is advantageous. The neuroprotective effects of serine-proteases have not been so far recognized.

Protein C (PC) is a member of the class of vitamin K-dependent serine protease coagulation factors. Unlike the majority of coagulation factors, such as Factors VIIa, IXa, Xa, XIIa, thrombin, plasmin or plasma kallikrein which are procoagulants, Protein C regulates blood coagulation by acting as a natural anticoagulant that circulates in the blood in an inactive form that requires proteolytic activation to generate the anticoagulant enzyme. The activated form of Protein C, APC, inhibits blood coagulation at the levels of Factors V and VIII in the clotting cascade.

Similar to most other zymogens of extracellular proteases and the above recited blood coagulation factors, Protein C has the core structure of the chymotrypsin family, having insertions and N-terminus extensions that enable regulation of the zymogen and the

enzyme (See Owen W., in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, Colman et al., eds, pp. 235-241, J.B. Lippincott Co. (Philadelphia), 1987).

Protein C is composed of domains with discrete structure and function (See Foster et al., *Proc. Natl. Acad. Sci. USA.* 82:4673-4677 (1985) and Plutzky et al., *Proc. Natl. Acad. Sci. USA*, 83:546-550 (1986)). The light chain contains an amino-terminal gamma-carboxyglutamic acid (Gla) region, which is followed by two domains that are homologous to domains in the epidermal growth factor (EGF) precursor. The serine protease activity resides in the heavy chain.

The zymogen is activated by the action of thrombin at the site between the arginine residue at position number 15 of the heavy chain and the leucine residue at position 16 (chymotrypsin numbering) (See Kisiel, *J. Clin. Invest.*, 64:761-769, (1976); Marlar et al., *Blood*, 59:1067-1072 (1982); Fisher et al. *Protein Science*, 3:588-599 (1994)). Other proteins including Factor Xa (Haley et al., *J. Biol. Chem.*, 264:16303-16310 (1989), Russell's viper venom and trypsin (Esmon et al., *J. Biol. Chem.*, 251:2770-2776 (1976) have also been shown to enzymatically cleave and convert inactive protein C to its activated form. Activated protein C (APC) hydrolyzes arginine esters and related substrates via a core triad of catalytic amino acid residues that occur at Ser-195, His-57, and Asp-102 of the heavy chain (chymotrypsin numbering). The enzyme's specificity is restricted to a small number of protein substrates; blood coagulation cofactors, activated Factors V and VIII, as well as Factors V and VIII are the known macromolecular substrates for the proteolytic inactivation by activated protein C (See Kisiel et al., *Biochem.*, 16:5824-5831 (1977); Vehar et al., *Biochem.*, 19:401-410 (1980); and Walker et al., *Biochim. Biophys. Acta.*, 571:333-342 (1979)).

Thrombin, thought to be the major physiological protein C activator, activates protein C slowly in purified systems, plasma, or blood, when in the presence of physiological concentrations of calcium. A membrane-bound thrombin receptor called thrombomodulin has been identified which accelerates protein C activation. Thrombin binds to thrombomodulin on the luminal surface of endothelial cells and undergoes an increase in specificity for protein C. Calcium is required for this process. Additional studies have revealed that the membrane-lipid binding domain of protein C, the vitamin-K

dependent Gla domain, is also required for normal activation of protein C (Esmon et al., in "Progress in Vascular Biology, Hemostasis, and Thrombosis", Ruggeri et al., eds., *Annals of The New York Academy of Sciences*, Vol. 614:30-43 (1991)). Endothelial protein C receptor (EPCR) enhances protein C activation by thrombin bound to thrombomodulin.

Thrombosis and thromboembolism, the occurrence of occlusive thrombi in the vasculature of human patients, poses a significant clinical problem and is a significant cause of morbidity and mortality. Arterial thrombi are responsible for myocardial infarction (MI) and cerebral ischemia (stroke), while venous thrombi cause deep vein thrombosis (DVT) and pulmonary embolism (PE). The magnitude of the clinical challenge created by thrombi is reflected in morbidity and mortality statistics. One of the leading causes of death in men over the age of 50 is acute MI, and stroke remains a debilitating and unpredictable disease.

Deep vein thrombosis is a common disease. Well established risk factors include recent surgery, malignant disorders, pregnancy and labor, long term immobilization, and deficiency of one of the main inhibitors of the clotting system. The main inhibitors are known to be protein C, protein S and antithrombin. The causes of deep vein thrombosis in many patients remain unclear. It has recently been established however that a poor anticoagulant response to activated protein C (APC) is present in many families with a hereditary tendency to venous thrombosis.

Inflammation is the body's reaction to injury and infection. Three major events are involved in inflammation: (1) increased blood supply to the injured or infected area; (2) increased capillary permeability enabled by retraction of endothelial cells; and (3) migration of leukocytes out of the capillaries and into the surrounding tissue (hereinafter referred to as cellular infiltration) (Roitt et al., *Immunology*, Grower Medical Publishing, New York, 1989).

Increased capillary permeability allows larger molecules to cross the endothelium that are not ordinarily capable of doing so, thereby allowing mediators of immunity such as leukocytes to reach the injured or infected site. Leukocytes, primarily neutrophil polymorphs (also known as polymorphonuclear leukocytes, neutrophils or PMN) and macrophages, migrate to the injured site by a process known as chemotaxis. At the site of

inflammation, tissue damage and complement activation cause the release of chemotactic peptides, such as C5a. Complement activation products are also responsible for causing degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction and increases in vascular permeability (Mulligan et al. *J. Immunol.* 148:1479-1485 (1991)).

Although leukocyte traversal of vessel walls to extravascular tissue is necessary for host defense against foreign antigens and organisms, leukocyte-endothelial interactions often have deleterious consequences for the host. For example, during the process of adherence and transendothelial migration, leukocytes release oxidants, proteases and cytokines that directly damage endothelium or cause endothelial dysfunction. Once at the extravascular site, emigrated leukocytes further contribute to tissue damage by releasing a variety of inflammatory mediators. Moreover, single leukocytes sticking within the capillary lumen or aggregation of leukocytes within larger vessels are responsible for microvascular occlusion and ischemia. Leukocyte-mediated vascular and tissue injury has been implicated in pathogenesis of a wide variety of clinical disorders, such as acute and chronic allograft rejection, vasculitis, rheumatoid and other forms of inflammatory based arthritis, inflammatory skin diseases, adult respiratory distress syndrome, ischemia-reperfusion syndromes such as myocardial infarction, shock, stroke, organ transplantation, crush injury and limb replantation.

Many other serious clinical conditions involve underlying inflammatory processes in humans. For example, multiple sclerosis (MS) is an inflammatory disease of the central nervous system. In MS, circulating leukocytes infiltrate inflamed brain endothelium and damage myelin, with resultant impaired nerve conduction and paralysis (Yednock et al., *Nature* 366:63-66 (1992)). Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of tissue damage caused by self antigen directed antibodies. Auto-antibodies bound to antigens in various organs lead to complement-mediated and inflammatory cell mediated tissue damage (Theofilopoubs, A.N., *Encyclopedia of Immunology*, pp. 1414-1417 (1992)).

Reperfusion injury is another condition associated with activation of the inflammatory system and enhanced leukocyte-endothelial cell (EC) adhesion. There is

much evidence that adhesion-promoting molecules facilitate interactions between leukocytes and endothelial cells and play important roles in acute inflammatory reaction and accompanying tissue injury. For example, in acute lung injury caused by deposition of IgG immune complexes or after bolus i.v. infusion of cobra venom factor (CVF), neutrophil activation and the generation of toxic oxygen metabolites cause acute injury (Mulligan et al., *J. Immunol.* 150(6):2401-2405 (1992)). Neutrophils (PMNs) are also known to mediate ischemia/reperfusion injury in skeletal and cardiac muscle, kidney and other tissues (Pemberton et al., *J. Immunol.* 150:5104-5113 (1993)).

Infiltration of airways by inflammatory cells, particularly eosinophils, neutrophils and T lymphocytes, is a characteristic feature of atopic or allergic asthma (Cotran et al., *Pathological Basis of Disease*, W. B. Saunders, Philadelphia, 1994). Cellular infiltration of the pancreas with resultant destruction of islet beta-cells is the underlying pathogenesis associated with insulin-dependent diabetes melitis (Burkly et al., *Diabetes* 43: 529-534 (1994)). Activation of inflammatory cells whose products cause tissue injury underlies the pathology of inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis (Cotran et al., 1994). Neutrophils, eosinophils, mast cells, lymphocytes and macrophages contribute to the inflammatory response. Minute microabcesses of neutrophils in the upper epithelial layers of the dermis accompany the characteristic epidermal hyperplasia/thickening and scaling in psoriasis.

Various anti-inflammatory drugs are currently available for use in treating conditions involving underlying inflammatory processes. Their effectiveness however, is widely variable and there remains a significant clinical unmet need. This is especially true in the aforementioned diseases where available therapy is either of limited effectiveness or is accompanied by unwanted side effect profiles. Moreover, few clinical agents are available which directly inhibit cellular infiltration, a major underlying cause of tissue damage associated with inflammation. Thus, there is a need for a safe, effective clinical agent for preventing and ameliorating cellular infiltration and consequential pathologic conditions associated with inflammatory diseases, injuries and resultant perturbations of cytokine networks.

Therefore, there is a need in the art for new and better compounds and methods of their use in treating diseases associated with inflammation, thrombosis, and a variety of types of neurological damage.

SUMMARY OF THE INVENTION

The present invention overcomes many of these problems in the art by providing, in a first embodiment, methods for reducing inflammation in a subject having or at risk of having inflammatory vascular disease. The method includes administering to the subject, an anti-inflammatory effective amount of activated protein C (APC), thereby reducing inflammation in the subject.

In another embodiment, the invention provides methods for protecting neuronal cells from cell death in a subject having or at risk of having a neuropathological disorder by administering to the subject a neuroprotective effective amount of activated protein C (APC), thereby providing neuroprotection to the subject.

In yet another embodiment, the invention provides a method for reducing inflammation in a subject having or at risk of having a neuropathological disorder. The method includes administering to the subject, an anti-inflammatory effective amount of activated protein C (APC), thereby reducing neurological inflammation in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are graphs showing changes in cerebral blood flow (CBF) during MCA occlusion and reperfusion in control mice (Figure 1A) and APC-treated mice (Figure 1B). Time period (I) corresponds to CBF values taken after IV administration of either vehicle (Figure1A) or APC (Figure 1B) prior to occlusion. Values are expressed as mean ± SD, n = 6 for control group and n = 7 for APC-treated group. Significant differences in CBF values between the two groups are indicated by a star (*) ($p < 0.02$ to < 0.005).

Figures 2A and 2B are graphs showing brain injury determined by TTC staining in control and APC-treated mice subjected to 1 hr of transient MCA occlusion. Figure 2A

shows the volumes of brain infarction (left) and edema (Swanson correction, right). Values are mean ± SE, n = 6 for control mice, and n = 7 for APC-treated mice. *$p < 0.01$ and **$p < 0.05$ by Student's t-test. Figure 2B shows the infarct area for each of the five coronal sections of the same brain as in Figure 2A. Values are mean ± SE; * $p < 0.05$.

Figures 3A and 3B are schematic drawings showing the incidence and topography of the infarction at the level of the optic chasm during transient MCA occlusion in control mice (Figure 3A) and APC-treated mice (Figure 3B). The number of control mice and APC-treated mice was 6 and 7 respectively. Key for the incidence of topography (regions involved) is given in Figure 3A.

Figures 4A through 4D are a series of graphs showing fibrin-positive microvessels (Figures 4A and 4C), neutrophils (Figure 4B) and hemoglobin levels (Figure 4D) in the ischemic and contralateral hemisphere in control mice and APC-treated mice determined after 1 hr of transient MCA occlusion. Fibrin positive vessels and leukocytes were detected by immunostaining with anti-fibrin II antibody and CD11b antibody and dichloroacetate staining respectively. Hemoglobin levels in hemispheric sections were determined by spectrophotometric hemoglobin assay. Values are mean ± SE. n = 6 for control mice (open bars) and n = 7 for APC-treated mice (closed bars).

Figures 5A and 5B are graphs showing Western blot detection of fibrin in brain sections of control and APC-treated mice with anti-fibrin NYB-T2G1 antibody. Figure 5A shows signal from Western blot standard curve samples was linear in the range between 0.05 and 3 μg/0.1 ml. Figure 5B shows Western blot analysis of 10 mg brain tissue sections at the level of the optic chiasma in control and APC-treated mice in the ischemic and contralateral hemispheres. Fibrin levels were determined by scanning densitometry using the standard curve (mean ± SE, n = 3).

Figures 6A through 6E are graphs showing brain infarction area (Figure 7A) and edema volume (Figure 6B), cerebral blood flow (CBF) during reperfusion (Figure 6C), neutrophils (Figure 6D) and fibrin-positive vessels (Figure 6E) in ischemic hemisphere in mice treated with vehicle or APC after stroke induction. Vehicle (open bars), or APC (2 mg/kg, closed bars), 0.5 mg/kg (light gray bars) and 0.1 mg/kg (dark gray bars) were

given 10 min after the MCA occlusion. Mean ± SE, from 3 to 5 animals. *$p < 0.05$ and **$p < 0.01$.

Figure 7 is a graph showing the percentage of ICAM-1-positive vessels (mean ± SE) in control (n = 4; open bars) and APC-treated mice (n = 4; closed bars) after treatment with 2 mg/kg APC given 10 min after the MCA occlusion.

Figures 8A and 8B are graphs showing infarction area (Figure 8A) and edema volume (Figure 8B) in the ischemic hemisphere in mice after stroke induction treated with vehicle alone (control), low dose (0.1 mg/kg) of APC alone, low dose APC coinjected with protein S (2 mg/kg) or protein S alone. Mean ± SE, from 2 to 5 animals. *$p<0.05$ and ns = non-significant.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the seminal discovery that APC has significant - neuroprotective, antithrombotic and anti-inflammatory effects of APC in a murine model of transient focal cerebral ischemia with cerebrovascular thrombosis whether administered before or after the ischemic event. In this model, reductions in the cerebral blood flow (CBF) during middle cerebral artery (MCA) occlusion and reperfusion in control animals were more pronounced than in previously reported murine stroke models, i.e., by about 2 to 3-fold, respectively, leading to large brain infarcts, swelling, development of significant inflammatory response, thrombosis and death relatively early during reperfusion. Treatment with APC either before or after induction of stroke protected mice from accelerated stroke-related death and restored CBF almost completely during MCA reperfusion. These effects were associated with significant reductions in the extent of brain injury and swelling, and improvement in motor neurological performance. While not wanting to be bound to a particular theory, it is believed that the neuroprotective effects of APC are related to its anti-inflammatory activities, as suggested by the remarkable reduction in number of PMNs that infiltrate the ischemic hemisphere (due to prevention of their migration across the blood-brain barrier), and also due to its antithrombotic effects, as suggested by the significant reduction in cerebrovascular fibrin deposits in stroke-induced animals.

The beneficial effects of APC in the present invention were associated with marked improvement of post-ischemic re-circulation, i.e., 79% of baseline CBF values in comparison to only 32% in control animals. It is possible that the observed CBF improvement involves, in part, alleviation of post-ischemic coagulopathy by APC. Previous studies of global ischemia revealed massive intravascular coagulation in association with complement activation. Studies in focal ischemia models also revealed that significant obstructions in CBF might result from massive microvascular occlusions due to vascular accumulation of polymorphonuclear (PMNs) leukocytes and fibrin deposition. Animals which lack a key fibrinolytic factor, for example tPA-/- mice, may develop substantial ischemic brain thrombosis and injury even when the CBF was only moderately reduced. While previous studies involving organs other than brain reported significant anticoagulant activity of APC in vitro and in vivo in different microarterial thrombosis models, the present study demonstrates not only anticoagulant effects but also anti-inflammatory and neuroprotective effects of APC.

Given the data provided in the Examples described herein, it is believed that anti-inflammatory effects of APC contribute to restoration of post-ischemic CBF. The Examples show that APC, in addition to significantly reducing fibrin deposition in the ischemic hemisphere, also prevents intravascular accumulation of peripheral blood cells, i.e., PMNs, in brain by preventing their transport across the blood-brain barrier. Although the mechanisms of anti-inflammatory effects of APC are still not completely understood, the absence of significant leukocyte-endothelial interactions in ischemic APC-treated animals may not only reduce fibrin formation in ischemic brain, but could also be related to improvements in the CBF and reduced neuronal injury. It has been shown that blocking PMN penetration across the blood-brain barrier results in considerable improvement of the neurological outcome and also limits neuronal injury.

The inventors now believe that one of the mechanisms by which APC minimizes damage in both the cerebral vascular system and brain is by reducing inflammatory reactions. The present study demonstrated that APC prevented stroke-related death, reduced volumes of brain infarction and edema by 59% and 50%, respectively, and improved motor neurologic score by 2-fold. It is likely that the increased velocity of post-ischemic re-circulation results in faster restoration of energy-producing metabolites,

recovery of the redox state of the respiratory chain and restoration of normal tissue energy state with reactivation of ion exchange pumps, which are pivotal for normal neuronal functioning and elimination of post-ischemic edema. The capability of APC to abolish almost completely leukocyte accumulation within the vascular system and prevent PMN penetration into brain parenchyma may be responsible, in part, for its neuroprotective effects. This may increase not only the rate of re-circulation, but may also importantly alleviate post-ischemic generation of reactive oxygen species from PMNs, which in turn may protect neurons from injury. Although the neuroprotective effects of APC in stroke can be rationalized by its anti-inflammatory action and antithrombotic effects, we cannot rule out the possibility that APC itself may also have direct neuroprotective effects on neurons.

The present study indicated that APC does not adversely affect hemostatic function or produce increased bleeding in the brain or intracerebral hemorrhage (ICH). This confirms findings from previous experimental studies with APC in demonstrating that elevated levels of APC appear not to cause bleeding. In contrast to APC, bleeding and ICH were reported as potential life-threatening complications with other forms of antithrombotic therapy for stroke including thrombolytic treatment with plasminogen activators (e.g., tPA) or anticoagulant treatment with heparin.

Prospective epidemiological studies have suggested that endogenous protein C zymogen may be protective in stroke in humans. Low levels of plasma protein C or APC, and/or resistance to the anticoagulant effects of APC were related to poor outcome after stroke. Low plasma levels of protein C observed in stroke patients may be caused by lower levels of protein C biosynthesis and/or by protein C depletion due to excessive thrombin generation and rapid APC clearance, while low circulating APC may result from depletion of protein C zymogen precursor, increased levels of circulating APC inhibitors, or reduced APC generating capacity due to either low levels of intravascular thrombin, or reduced thrombomodulin and/or endothelial cell protein C receptor. It has been speculated that generation of APC from ischemic tissues is protective, for example during cerebral ischemia in humans and after cardiopulmonary bypass surgery. Results presented here give insights into previous clinical studies and suggest the potential relevance of APC as a

neuroprotective agent with multiple actions that may be beneficial for clinical applications in stroke.

Neurodegenerative diseases include diseases in which neuronal cells degenerate to bring about a deterioration of cognitive functions. A variety of diseases and neurological deficiencies may bring about the degeneration of neuronal cells, including Alzheimer's disease, Huntington disease or chorea, hypoxia or hlischemia caused by stroke, cell death caused by epilepsy, amyotrophic lateral sclerosis, mental retardation and the like, as well as neurodegenerative changes resulting from aging.

In a first embodiment, the invention provides methods for reducing inflammation in a subject having or at risk of having inflammatory vascular disease. The method includes administering to the subject, an anti-inflammatory effective amount of activated protein C (APC), for example, in a pharmaceutically acceptable carrier, thereby reducing inflammation in the subject.

The present invention is useful for treating many clinical conditions involving inflammatory processes. For example, inflammatory bowel diseases including Crohn's disease and ulcerative colitis are spontaneous chronic inflammations of the gastrointestinal tract which involve activation of inflammatory cells whose products cause tissue injury. Neutrophils, eosinophils, mast cells, lymphocytes and macrophages contribute to the inflammatory response. While not wanting to be bound to a particular theory, it is speculated that APC acts on brain endothelial cells (i.e., vascular cells), via an endothelial receptor for protein C and APC, that mediates its effects on endothelium, both central and peripheral. This, in turns affects intracellular signaling systems that in a cascade turn on and off different genes in vascular endothelium that may interfere with normal endothelial cell response to inflammation. For example, it is known that adhesion molecules mediate the interactions of leucocytes with the vessel wall. It is possible that APC prevents their expression and this is why the inflammatory response is inhibited. Adhesion molecules that may mediate this response may include molecules such as ICAM, VCAM, or PECAM.

The present invention is also directed to treatment of systemic shock and many resultant clinical conditions associated therewith. Systemic shock often occurs as a

complication of severe blood loss, severe localized bacterial infection and ischemia/reperfusion trauma and it is a major cause of death in intensive care units. Most cases of septic shock are induced by endotoxins (i.e., bacterial cell wall lipopolysaccharides or LPS) from gram negative bacilli or toxins (i.e., toxic shock toxin 1) from gram positive cocci bacteria. The release of LPS in the bloodstream causes release of inflammatory mediators (inflammatory cytokines, platelet activating factor, complement, leukotrienes, oxygen metabolites, and the like) which cause myocardial dysfunction, vasodilation, hypotension, endothelial injury, leukocyte adhesion and aggregation, disseminated intravascular coagulation, adult respiratory distress syndrome (ARDS), liver, kidney and central nervous system (CNS) failure. Shock due to blood loss also involves inflammatory mediator release. In each case, inflammatory responses are induced at the original site of trauma, and also in the vasculature and remote vascularized sites.

Myocardial ischemia is associated with activation of the complement system which further promotes cardiac injury with the enhancement of a series of inflammatory events. Life threatening local and remote tissue damage occurs during surgery, trauma and stroke when major vascular beds are deprived for a time of oxygenation (ischemia), then restored with normal circulation (reperfusion). Reperfusion injury is characterized by vascular permeability leading to edema and infiltration of inflammatory cells. Neutrophils contribute significantly to reperfusion damage by generating oxidants or releasing proteases that damage the microvasculature or adjacent tissue. Cell death and tissue damage due to complement and inflammatory cell mechanisms lead to organ failure or decreased organ function. The activation of mediators by a local injury can also cause a remote injury to highly vascularized organs. The compositions and methodologies of the present invention are useful in the treatment of ischemia and reperfusion injury.

Inflammatory response damage also occurs in glomerulonephritis as well as tubule disease. Infiltration of inflammatory cells (especially macrophages) is linked to proteinuria accompanied histologically by hypercellularity and crescent formation in glomeruli. Over a longer term, the infiltration of inflammatory cells is associated with accumulation of extracellular matrix and sclerosis and chronic compromise of renal function. The present invention is also efficacious in treating glomerulonephritis and tubule disease.

There are many other disease and injury conditions which benefit from the methodologies of the present invention such as for example, coronary arterial occlusion, cardiac arrhythmias, congestive heart failure, cardiomyopathy, bronchitis, acute allergic reactions and hypersensitivity, neurotrauma, graft/transplant rejection, myocarditis, insulin dependent diabetes, and stroke. Stroke involves a very strong inflammatory response, that in part may be responsible for neuronal damage directly by allowing leukocytes to enter the brain and destroy normal brain cells and neurons, and indirectly by obstructing microvessels and stopping blood flow. This again requires adhesion molecules and cytokines that may be direct or indirect targets of APC cellular interactions that are independent of its anticoagulant effects.

In addition to treating patients suffering from the trauma resulting from heart attack, patients suffering from actual physical trauma could be treated with APC in order to relieve the amount of inflammation and swelling which normally result after an area of the body is subjected to severe trauma. Also, patients suffering from hemorrhagic shock could be treated to alleviate inflammation associated with restoring blood flow. Other disease states which might be treatable using formulations of the invention include various types of arthritis, various chronic inflammatory conditions of the skin, insulin-dependent diabetes, and adult respiratory distress syndrome. After reading the present disclosure, those skilled in the art will recognize other disease states and/or symptoms which might be treated and/or mitigated by the administration of APC formulations of the present invention.

Some examples of arterial thrombosis where APC alone or in combination with a thrombolytic agent, anticoagulant, or anti-inflammatory is useful include the following clinical settings: 1) Acute arterial thrombotic occlusion including coronary, cerebral or peripheral arteries; 2) Acute thrombotic occlusion or restenosis after angioplasty; 3) Reocclusion or restenosis after thrombolytic therapy. Thrombolytic agents such as t-PA salvage ischemic tissue when used within hours of acute heart attack or stroke by re-establishing blood flow in the occluded artery. At present, between one-four and one-third of patients who have successful thrombolytic reperfusion of occluded coronary arteries subsequently undergo reocclusion after discontinuing t-PA infusion. This complication occurs despite full-dose heparin therapy. APC will have greater efficacy than heparin in

preventing reocclusion. 4) Small and large caliber vascular graft occlusion. Vascular grafts of small caliber, i.e., 3-/mm diameter, have a high frequency of thrombotic occlusion. APC alone or in combination with a thrombolytic agent is useful to prevent occlusion. 5) Hemodialysis. The prosthetic surfaces and flow design of all hemodialyzers are thrombogenic. Currently heparin is infused during dialysis. However, heparin is only partially effective, thereby limiting the reuse of dialyzers. Also, heparin has a number of troublesome side effects and complications. 6) Cardiopulmonary bypass surgery. To prevent thrombus formation in the oxygenator and pump apparatus, heparin is currently used. However, it fails to inhibit platelet activation and the resultant transient platelet dysfunction which predisposes to bleeding problems post-operatively. 7) Left ventricular cardiac assist device. This prosthetic pump is highly thrombogenic and results in life threatening thromboembolic events--complications that are only partially reduced by conventional anticoagulants (heparin or coumarin drugs). 8) Total artificial heart and left ventricular assist devices. 9) Other arterial thrombosis. APC is useful for arterial thrombosis or thromboembolism where current therapeutic measures are either contraindicated or not effective. For example, APC is useful for the treatment of acute pre- or postcapillary occlusion, including transplantations, retina thrombosis, or microthrombotic necrosis of any organ complicating infections, tumors, or coumarin treatment.

In another embodiment, the present invention provides methods for protecting neuronal cells from cell death in a subject having or at risk of having a neuropathological disorder is provided. The method includes administering to the subject, a neuroprotective effective amount of activated protein C (APC), for example, in a pharmaceutically acceptable carrier, thereby providing neuroprotection to the subject. Examples of "neuropathological disorders" include but are not limited to stroke, Alzheimer's disease, Huntington disease, ischemia, epilepsy, amyotrophic lateral sclerosis, mental retardation and aging. One "having or at risk of having" an inflammatory vascular disease as described herein is a subject either exhibiting symptoms of the disease or diagnosed as being at risk for developing the disease. Such subjects include those subjects having undergone or preparing for surgical procedures as described below.

In yet another embodiment, the invention provides methods for reducing inflammation in a subject having or at risk of having a neuropathological disorder. The method includes administering to the subject, an anti-inflammatory effective amount of activated protein C (APC), for example in a pharmaceutically acceptable carrier, thereby reducing neurological inflammation in the subject. The methodologies of the present invention are also efficacious in the treatment of multiple sclerosis (MS) in addition to the neuropathologies described above. MS is often characterized by the penetration of the blood-brain barrier by circulating leukocytes, leading to demyelination in various parts of the brain, impaired nerve conduction and, ultimately, paralysis.

The term "treatment" or "ameliorate" refers to reducing the symptoms of the disease, such as inflammation. The term "treatment" or "ameliorate" denotes a lessening of the detrimental effect of the inflammatory or neurological disease in the subject receiving therapy. The term "treatment" when referring to neurological disease used hereinafter does not necessarily mean that the neurodegenerative disease is completely eliminated, but rather that the cognitive facilities damaged by the disease are improved. "Therapeutically effective" as used herein, refers to that amount of APC that is of sufficient quantity to ameliorate the cause or symptoms of the disease. The subject of the invention is preferably a human, however, it can be envisioned that any animal in need of anti-inflammatory or neuroprotection can be treated using the methods of the invention.

The term "neurodegenerative disease" is used hereinafter to denote conditions which result in degeneration of neural cells in the brain which may bring about deterioration of cognitive function. Such degeneration of neural cells may be caused by Alzheimer's disease (e.g., Alzheimer's disease is characterized by synaptic loss and loss of neurons) Huntington disease or chorea; by pathological conditions caused by temporary lack of blood or oxygen supply to the brain, e.g., brought about by stroke; by epileptic seizures; due to chronic conditions such as amyotrophic lateral sclerosis, mental retardation; as well as due to normal degeneration due to aging. It should be noted that diseases such as stroke and Alzheimer's have both a neurodegenerative and an inflammatory vascular component and thus are treatable by the methods of the invention.

One aspect of the invention includes the "neuroprotective" activity of APC. The term "neuron" includes hundreds of different types of neurons, each with distinct properties. Each type of neuron produces and responds to different combinations of neurotransmitters and neurotrophic factors. Neurons are thought not to divide in the adult brain, nor do they generally survive long in vitro. The method of the invention provides for the protection from death or senescence of neurons from virtually any region of the brain and spinal cord. Neurons include those in embryonic, fetal or adult neural tissue, including tissue from the hippocampus, cerebellum, spinal cord, cortex (e.g., motor or somatosensory cortex), striatum, basal forebrain (cholenergic neurons), ventral mesencephalon (cells of the substantia nigra), and the locus ceruleus (neuroadrenaline cells of the central nervous system).

Whether in the brain or other tissue, APC acts on brain endothelial cells (i.e., vascular cells), via endothelial receptor(s) for protein C and APC, that mediates its effects on endothelium, both central and peripheral. This, in turns affects intracellular signaling systems that in a cascade turn on and off different genes in vascular endothelium that may interfere with normal endothelial cell response to inflammation. It is also possible that receptors for APC on neurons may mediate neuroprotective effects of APC.

Thus, in one aspect of the invention, it may be desirable to up-regulate the expression of such receptors in order to more effectively treat a particular disease.

The present invention is useful for treating many clinical conditions involving inflammatory processes. For example, inflammatory bowel diseases including Crohn's disease and ulcerative colitis are spontaneous chronic inflammations of the gastrointestinal tract which involve activation of inflammatory cells whose products cause tissue injury. Neutrophils, eosinophils, mast cells, lymphocytes and macrophages contribute to the inflammatory response.

In addition to administering APC to a subject as described herein, it may be desirable to co-administer one or more anti-inflammatory agent or additional neuroprotective agent. Co-administration may include administration prior to APC, simultaneously with APC or following APC administration.

Non-limiting examples of neuroprotective agents include N-methyl-D-aspartate (NMDA) receptor antogonists and calcium ion channel antagonists, such as are known in the art, and the like.

Examples of anti-inflammatory agents include but are not limited to: Aminoarylcarboxylic Acid Derivatives such as Etofenamate, Meclofenamic Acid, Mefanamic Acid, Niflumic Acid, Arylacetic Acid Derivatives such as Acemetacin, Amfenac, Cinmetacin, Clopirac, Diclofenac, Fenclofenac, Fenclorac, Fenclozic Acid, Fentiazac, Glucametacin, Isoxepac, Lonazolac, Metiazinic Acid, Oxametacine, Proglumetacin, Sulindac, Tiaramide, Tolmetin, Arylbutyric Acid Derivatives such as Butibufen, Fenbufen, Arylcarboxylic Acids such as Clidanac, Ketorolac, Tinoridine, Arylpropionic Acid Derivatives such as Bucloxic Acid, Carprofen, Fenoprofen, Flunoxaprofen, Ibuprofen, Ibuproxam, Oxaprozin, Piketoprofen, Pirprofen, Pranoprofen, Protizinic Acid, Tiaprofenic Acid, Pyrazoles such as Mepirizole, Pyrazolones such as Clofezone, Feprazone, Mofebutazone, Oxyphenbutazone, Phenylbutazone, Phenyl Pyrazolidininones, Suxibuzone, Thiazolinobutazone, Salicylic Acid Derivatives such as Bromosaligenin, Fendosal, Glycol Salicylate, Mesalamine, 1-Naphthyl Salicylate, Olsalazine, Sulfasalazine, Thiazinecarboxamides such as Droxicam, Isoxicam, Piroxicam. Others such as epsilon-Acetamidocaproic Acid, S-Adenosylmethionine, 3-Amino-4-hydroxybutyric Acid, Amixetrine, Bendazac, Bucolome, Carbazones, Difenpiramide, Ditazol, Guaiazulene, Heterocylic Aminoalkyl Esters of Mycophenolic Acid and Derivatives, Nabumetone, Nimesulide, Orgotein, Oxaceprol, Oxazole Derivatives, Paranyline, Pifoxime, 2-substituted-4, 6-di-tertiary-butyl-s-hydroxy-1,3-pyrimidines, Proquazone, Sialyl Lewis.sup.x Dimers, or Tenidap. Additional therapeutic agents which can be administered include steroids (e.g., glucocorticoids such as prednisone, methyl prednisolone and dexamethasone).

In addition to administering APC to a subject as described herein, it may be desirable to co-administer an anticoagulant, anti-platelet or thrombolytic agent. Co-administration may include administration prior to APC, simultaneously with APC or following APC administration. Examples of thrombolytic agents include but are not limited to urokinase, tPA, Lys-plasminogen, streptokinase, tissue plasminogen activator, prourokinase, acylated form of plasminogen, acylated form of plasmin, and acylated

streptokinase-plasminogen complex or any analogs thereof. Examples of anticoagulants include warfarin and heparin. Further, anticoagulant antibodies, such as those described in US Patent No. 5,679,639, incorporated by reference, can be co-administered with APC. Anti-platelet agents include, for example, aspirin, dipyridamole, clopidogrel, abciximab (Reopro) or any inhibitor of plately glycoprotein IIb-IIIa.

Compositions utilized in the present inventions and methods of preparation and administration include those described in US Patent Nos . 5, 084, 274, 6, 037,322 and 6,156,734, which are herein incorporated by reference each in its entirety. "Activated Protein C" refers to Protein C that is cleaved proteolytically by thrombin to yield an activated protein C (APC) which inactivates coagulation Factors Va and VIIIa thus inhibiting coagulation. The methods of use of APC described herein include " fragments of APC, as long as they retain the activities described herein. Such fragments, or APC, include recombinantly produced, human plasma-derived and synthetically produced, for example, as well as derivatives thereof. "Synthetic peptide" refers to a chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof. "Anticoagulant" refers to an agent that interrupts coagulation and thereby inhibits fibrin formation. "Coagulation" refers to the sequential process in which the multiple coagulation factors of the blood interact resulting in the formation of fibrin. Protein C consists of a 155 amino acid residue light chain and a 262 amino acid residue heavy chain and is fully described in US Patent No. 5,679,639 herein incorporated by reference.

"Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino

acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. APC of the present invention also include any polypeptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity, e.g., anti-inflammatory or neuroprotective, is maintained.

Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with APC, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

A therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

APC is a very species specific moiety. The dosage for human or recombinant human APC in a human is much lower than the appropriate dosage in a mouse, for example. The normal baseline level of APC in a human is typically about 2.2 ng/ml of blood. In practice of the invention methods, it is necessary to administer sufficient APC to raise the blood level slightly above the baseline level, but not so much as to risk causing undesireable bleeding. A therapeutically effective amount of human APC is typically administered to a human at a dosage sufficient to raise the blood level of APC by from about 1.0 ng/ml to about 500 ng/ml, preferably, from about 5 ng/ml to about 200 ng/ml.

The APC can be formulated according to known methods to prepare pharmaceutically useful compositions. The APC is preferably administered parenterally to ensure its delivery into the bloodstream in an effective form by injecting the appropriate dose as continuous infusion for about 4 to about 96 hours. Preferably, the appropriate dose of APC will be administered by continuous infusion for about 4 to about 72 hours. More

preferably, the appropriate dose of APC will be administered by continuous infusion for about 4 to about 48 hours. More preferably, the appropriate dose of APC will be administered by continuous infusion for about 12 to about 48 hours. More preferably, the appropriate dose of APC will be administered by continuous infusion for about 12 to about 36 hours. More preferably, the appropriate dose of APC will be administered by continuous infusion for about 4 to about 36 hours. More preferably, the appropriate dose of APC will be administered by continuous infusion for about 12 to about 24 hours. Most preferably, the appropriate dose of APC will be administered by continuous infusion for about 24 hours. The administration of APC will begin as soon as possible following diagnosis of the vascular occlusive or arterial thromboembolic disorder. An appropriate loading dose of APC may be given by bolus injection with or without subsequent APC infusion.

The amount of APC administered can be from about 0.01 mg/kg/hr to about 0.10 mg/kg/hr which is equivalent to about 17 mg/70 kg/24 hours to about 170mg/70 kg/24 hours. While the dose level is identified as a specific amount per 24 hours, one skilled in the art would recognize that this is a designation of the dose level and is not necessarily limited to a 24 hour infusion but may include continuous infusion for various times, for example, from about four hours to about ninety-six hours. More preferably the amount of APC administered is about 0.01 mg/kg/hr to about 0.05 mg/kg/hr (about 17 mg/70 kg/24 hours to about 84 mg/70 kg/24 hours). While more preferably the amount of APC administered will be about 0.01 mg/kg/hr to about 0.03 mg/kg/hr (about 17 mg/70 kg/24 hours to about 50 mg/70 kg/24 hours). Furthermore, the amount of APC administered is from about 0.02 mg/kg/hr to about 0.05 mg/kg/hr which is equivalent to about 34 mg/70 kg/24 hours to about 84 mg/70 kg/24 hours. More preferably the amount of APC administered is about 0.024 mg/kg/hr to about 0.048 mg/kg/hr (about 40 mg/70 kg/24 hours to about 80 mg/70 kg/24 hours). While more preferably the amount of APC administered will be about 0.027 mg/kg/hr to about 0.045 mg/kg/hr (about 45 mg/70 kg/24 hours to about 75 mg/70 kg/24 hours). While more preferably the amount of APC administered will be about 0.030 mg/kg/hr to about 0.042 mg/kg/hr (about 50 mg/70 kg/24 hours to about 70 mg/70 kg/24 hours). While more preferably the amount of APC administered will be about 0.033 mg/kg/hr to about 0.039 mg/kg/hr (about 55 mg/70 kg/24

hours to about 65 mg/70 kg/24 hours). Preferable amounts of APC administered are about 0.024 mg/kg/hr (about 40 mg/70 kg/24 hours), about 0.027 mg/kg/hr (about 45 mg/70 kg/24 hours) or, about 0.030 mg/kg/hr to about 0.042 mg/kg/hr (about 50 mg/70 kg/24 hours). Clearly, the amount of APC can be reduced when administered with a co-factor such as Protein S, as described herein.

Alternatively, the APC will be administered by injecting a portion of the appropriate dose per hour as a bolus injection over a time from about 5 minutes to about 120 minutes, followed by continuous infusion of the appropriate dose for about twenty three hours to about 96 hours which results in the appropriate dose administered over 24 hours to 96 hours.

The most preferable dose level of APC to be administered for thrombotic occlusion (e.g. stroke) as described herein will be about 0.024mg/kg/hr.

A therapeutically effective amount of an anticoagulant antibody that may be used in conjunction with the methods of the invention is typically an amount of antibody such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram (ug) per milliliter (ml) to about 100 ug/ml, preferably from about 1 ug/ml to about 5 ug/ml, and usually about 5 ug/ml.

The therapeutic compositions containing APC are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle. The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the

route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

The pharmaceutical composition may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which may contain one or more accessory ingredients. In general, the composition for pills, tablets, or capsules (e.g., for oral administration) or powders are prepared by uniformly and intimately blending the compounds with finely divided solid carriers, and then, if necessary as in the case of tablets, forming the product into the desired shape and size. The pill, tablet, or capsule can be coated with a substance capable of protecting the composition from the gastric acid or intestinal enzymes in the subject's stomach for a period of time sufficient to allow the composition to pass undigested into the subject's small intestine. The pharmaceutical compositions may be also directly targeted to the brain by an intercerebroventricular pump.

Compositions suitable for parenteral administration (e.g., subcutaneous, intravenous, or intermuscular), on the other hand, conveniently comprise sterile aqueous solutions of the compound(s) in water or saline to produce an aqueous solution, and rendering said solution sterile. The composition may be presented in unit or multi-dose containers, for example, sealed ampoules or vials.

In another aspect, the invention provides methods for preventing neuronal death in a patient by administering to a patient a therapeutically effective amount of the pharmaceutical composition of the invention. The pharmaceutical composition of the invention can, for example, be used to treat acute traumatic events such as stroke, CNS trauma (e.g., brain or spinal surgery or injury), injury resulting from neurotoxins, and epilepsy; chronic neurodegenerative diseases such as Huntington's Chorea, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), AIDS-related neuronal degeneration, and

brain aging; or other neurodegenerative disorders related to the overactivation of the NMDA receptor.

In particular, it has been discovered that protein S, a co-factor of APC, has a synergistic effect when administered in accordance with the methods of this invention. For example, Example 3 below illustrates that administration of a combination of protein S and APC in the treatment of stroke in a mouse model is more effective for reducing the area of brain infarction and volume of edema in the ischemic hemisphere in mice than is a therapeutically effective low amount of APC administered alone, even when therapy is administered after the ischemic event. This unexpected result is obtained even when the amount of the APC administered (e.g., 0.1 mg/kg) in the combination therapy is far less than is generally used when APC alone is used in treatment of stroke in this model. Thus, a far lower amount of APC is an effective dose when the APC is administered in conjunction with protein S, i.e., simultaneously, before or after administration of the protein S. However, it should be noted that mice do not contain endogenous protein S; whereas normal humans do contain endogenous protein S. It is believed, therefore, that the presence of normal levels of endogenous protein S (e.g.,in humans) substantially lowers (perhaps by a factor as great as 100-fold) the therapeutic dosage of APC used in the invention methods. Further, it has been discovered that this synergistic effect of the combined presence of protein S and APC is also species specific, depending upon the APC and the cofactor being from the same species. For this reason, when non-human APC is used in treatment of humans, it is preferred to co-administer protein S derived from the same non-human species as the APC.

APC, analogs or biologically active fragments thereof may be administered in a manner compatible with the dosage formulation and in such amount as will be therapeutically effective. In particular, it has been discovered that invention treatment methods are equally effective whether the APC is administered during, before or after an ischemic event, such as stroke, e.g., up to three to six hours after a stroke.

The APC-containing compositions of the invention may be administered in any way which is medically acceptable which may depend on the disease condition or injury

being treated. Possible administration routes include injections, by parenteral routes such as intravascular, intravenous, intra-arterial, subcutaneous, intramuscular, intratumor, intraperitoneal, intraventricular, intraepidural or others, as well as oral, nasal, ophthalmic, rectal, topical, or by inhalation. The compositions may also be directly applied to tissue surfaces during surgery. Sustained release administration is also specifically included in the invention, by such means as depot injections or erodible implants.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLE 1

Animals.

Studies were performed in male C57BL/6 mice weighing 23-26 g using procedures approved by the Institutional Animal Care and Use Committee.. Mice were subjected to a modified middle cerebral artery (MCA) occlusion technique (E.S. Connolly et al., *Neurosurgery.* 38(3):523-531 (1996); H. Hara et al, *J. Cereb. Blood Flow Metab.*16:605-611, (1996); and P. Tabrizi et al., *Arterioscler. Thromb. Vasc. Biol.* 19:2801-2806 (1999)) to induce acute focal ischemic stroke with cerebrovascular thrombosis, as described below. Mice were initially anesthetized with metofane and maintained with 50 mg/kg i.p. pentobarbital. Atropine methyl nitrate (0.18 mg/kg i.p.) was given as pre-medication to prevent airway obstruction by mucus formation. Animals were allowed to breath spontaneously. Rectal temperature was maintained at $37 \pm 1\ ^{0}C$ by a thermostatically regulated heating pad. The right femoral artery was cannulated with a PE-10 catheter for continuous monitoring of blood pressure and blood analysis.

Preparation of stroke model.

A modified intravascular MCA occlusion technique (Connolly et al., 1996; Hara et al, 1996; and Tabrizi et al., 1999) was used to block the cerebral blood flow (CBF) almost completely in the MCA territory in the ischemic hemisphere. We used non-siliconized uncoated 6-0 8 mm long prolene suture with a modified rounded tip with a diameter of 0.20 mm. With this modification in the tip of the suture, CBF values dropped typically close to 10% of control baseline values immediately after placement of the suture in mice

weighing 23-26 g (see below). In contrast, previous studies used nylon filament coated with silicone that does not have thrombogenic properties (Y.R. Wang et al., *Nature Med.* 4:228-231 (1998)) and considered the procedure to be technically successful if ≥ 50% reduction in relative CBF was observed during the occlusion. The pronounced reduction in the CBF in the present model resulted in significant cerebrovascular thrombosis mainly in small microvessels, large infarcts and stroke-related death often relatively early in the reperfusion phase when CBF was reestablished by withdrawing the suture from cerebral arteries. Briefly, under the operating microscope, the right common carotid artery was exposed through a ventral midline incision, and the external carotid artery and its branches ligated. Through a transverse incision in the artery, the suture was introduced into the external carotid artery lumen, and gently advanced into the internal carotid artery to occlude the MCA at its origin from the circle of Willis. MCA occlusion was maintained for 1 hr followed by 24 hrs of reperfusion.

Blood flow and head temperature measurements.

CBF was monitored by Laser Doppler Flowmetry (LDF) using tissue perfusion monitor (Transonic BLF21, Ithaca, NY). CBF measurements were performed in all animals subjected to neuropathological and neurologic analysis. For CBF determinations, animals were placed in a stereotactic head frame, and readings were obtained 2 mm posterior to the bregma, both 3 and 6 mm to each side of midline, using a stereotactic micromanipulator and keeping the angle of the probe perpendicular to the cortical surface. LDF probes (0.8 mm diameter) positioned on the cortical surface were connected to a tissue perfusion monitor (Transonic BLF21). The procedure was considered to be technically successful if ≥ 88% reduction in relative CBF was observed immediately after placement of the occluding suture. The procedure was successful in all studied control animals producing levels of ischemia sufficient to render consistently large infarction volumes and microvascular thrombosis in the ischemic hemisphere. Head temperature was monitored with a 36-gauge thermocouple temperature probe in the temporalis muscle connected to a digital thermometer/thermoregulator model (model 9000, Omega, CT).

Survival time and neurologic deficits.

Survival time was monitored within 24 hrs of reperfusion and stroke-related deaths recorded. Neurologic examinations were performed at 24 hr after reperfusion and in some animals also at 3 hrs of reperfusion. Neurologic findings were scored on the following 5-point modified scale: no neurologic deficit (0); failure to extend left forepaw fully (1); turning to left (2); circling to left (3); unable to walk spontaneously (4); and stroke-related death (5) (Hara et al, 1996 and Tabrizi et al., 1999).

Blood analysis.

Arterial blood gases (pH, PaO_2, $PaCO_2$) were measured before and during MCA occlusion using ABL 30 Acid-Base Analyzer (Radiometer, Copenhagen, Denmark).

Measurement of volume of injury.

The area of injury was delineated by incubation of unfixed 1-mm coronal brain slices in 2% TTC in phosphate buffer (pH 7.4). Serial coronal sections were displayed on a digitizing video screen using the imaging system of Jandel Scientific (San Rafael, CA). The volume of injury was calculated by summing up affected areas from each coronal section and multiplying by the thickness of each section. Brain infarction and edema were calculated using Swanson correction (Tabrizi et al., 1999).

Histopathology.

Detection of fibrin by immunostaining was performed on brain tissue sections previously treated with TTC. Tissue fixed in 10% buffer formalin was processed and 4 μm thick paraffin coronal sections from each block cut and stained. Fibrin was localized using anti-fibrin II antibody (NYB-T2G1, Accurate Chemical Sci. Corp., Westbury, NY) (1:500 dilution) and graded according to the following scale (P. Tabrizi et al., 1999 and Y. Okada et al., *Stroke.* 25:1847-1853, 1994): 1, fibrin deposition limited to intravascular space; 2, fibrin deposition in the intravascular lumen and the perivascular space; 3, fibrin lattices in the extravascular or parenchymal tissue only. It has been previously shown that anti-human fibrin antibody cross reacts with mouse fibrin (Tabrizi et al., 1999 and H. Weiler-Guettler et al., *J. Clin. Invest.* 101:1983-1991 (1998)). All visual analyses were performed

by two observers blinded to the specimen source or timing. Paraffin sections were also stained using the anti-CD11b antibody (DAKO corporation, Carpenteria, CA) (1:250 dilution) directed against the leukocyte adhesion receptor macrophage-1 antigen. This antibody detects CD11b antigen on polymorphonuclear leucocytes (PMNs), as well as on activated macrophages and/or microglia in the tissue. The number of CD11b positive cells was counted in ten random fields by two independent blinded observers and expressed as number per mm^2 of section. Simultaneous detection of fibrin and leukocytes was done by sequential immunostaining. Fibrin was localized first with the NYB-T2G1 antibody and detected using 3, 3' diaminobenzidine (DAB) substrate (Vector Labs, CA) followed by detection of leukocytes using the CD11b antibody and the Vector SG peroxide substrate (Vector Labs, CA). Routine control sections included deletion of primary antibody, deletion of secondary antibody and the use of an irrelevant primary antibody. Neutrophils were also detected in brain sections by dichloroacetate esterase staining using the esterase staining kit (Sigma, MO) as reported (S.G. Soriano et al, *Stroke.* 30:134-139 (1999)). Sections were deparaffinized and incubated with naphthol AS-D chloroacetate. The free naphthol released on ester hydrolysis by enzymes on neutrophils was detected using freshly formed diazonium salt and counterstained with Gill No.3 hematoxylin. The number of neutrophils was counted as above.

Detection of fibrin in brain tissue sections by quantitative Western blot.

The procedure was as discussed previously (Tabrizi et al., 1999 and H. Weiler-Guettler et al., *J. Clin. Invest.* 101:1983-1991 (1998)). Briefly, after TTC staining, a 1 mm section of brain tissue was divided into contralateral and ipsilateral hemisphere. Tissue was homogenized in 10 mM sodium phosphate buffer, pH 7.5, 0.1 mM aminocaproic acid, 5 mM trisodium EDTA, 10 U aprotinin/ml, 10 U heparin/ml, and 2 mM PMSF. The homogenate was agitated for 14 hour at 4 ^{0}C, and the particulate material was sedimented by centrifugation at 10,000 g for 10 min, resuspended in extraction buffer without PMSF, sedimented again, and finally dispersed in 3 M urea. The suspension was agitated for 2 hours at 37 ^{0}C, vigorously vortexed, and centrifuged at 14,000 g for 15 min. The supernatant was aspirated and the sediment dissolved at 65 ^{0}C in reducing SDS buffer, subjected to SDS-PAGE (8%), and transferred to a PVDF membrane (Immobilon-P; Millipore Corp., MA) by electroblotting. Fibrin was visualized with anti-human fibrin II

antibody (given above) and enhanced chemiluminescence system (Amersham Corp., IL). Fibrin standards were prepared by clotting a known amount of murine fibrinogen (Sigma Chemical Co., MO) with an excess of thrombin in the absence of calcium. The films were scanned with a Hoefer GS 300 scanning densitometer interfaced to an IBM PC computer with a DT 2805 analog and digital system (Data Translation, MA) and data were converted into μg fibrin/0.1 g of tissue.

Spectrophotometric hemoglobin assay.

This assay was performed to detect microhemorrhages in the brain tissue. The procedure was as described previously (T.F. Choudhri et al.,*Stroke.* 28(11):2296-2302 (1997)). Briefly, a section of brain tissue 1 mm thick was divided into ipsilateral and contralateral hemisphere after TTC staining. Distilled water (200 μl) was added, tissue homogenized for 30 sec, sonicated on ice for 1 min, and centrifuged at 13,000 rpm for 30 min. Hemoglobin-containing supernatant was collected, and 80 μl of Drabkin's reagent (Sigma Diagnostics, MO) added to a 20-μl aliquot and allowed to mix for 15 min. This reaction converts hemoglobin to cyanmethemoglobin, which has an absorbency peak at 540 nm. To validate that the measured absorbency reflects the amount of hemoglobin, known quantities of bovine erythrocyte hemoglobin (Sigma) and incremental aliquots of mouse blood added to freshly homogenized brain tissue were analyzed in parallel.

Statistical analysis.

Physiological variables, infarction, and edema volumes were compared between groups using Student's t-test and ANOVA. Non-parametric data (neurologic outcome scores) was subjected to the Chi-square test with Fisher's transformation. Survival was compared by the Kruskal-Wallis test. A value of $p < 0.05$ was considered statistically significant.

Results.

There were no significant statistical differences in mean arterial blood pressure, PaO_2, $PaCO_2$, pH, hematocrit, head and body temperature, and blood glucose between control and APC-treated animals before MCA occlusion, during occlusion, and during

reperfusion (data not shown). No changes in head and body temperature were observed due to APC treatment during 15 min pre-MCA occlusion, and all blood parameters during that period were also within normal limits.

No significant differences in baseline tissue perfusion units were observed between control and APC-treated mice, indicating similar pre-occlusion CBF values. Baseline CBF readings were also taken before APC administration, and there was no significant difference with pre-occlusion values, indicating that APC did not influence the CBF under basal conditions. During MCA occlusion, the reductions in CBF in the ischemic hemisphere (point A) were significant in both groups; in the control group, the CBF dropped to between 9-13% of baseline values ($p < 0.001$; control MCA group) while in the APC-treated group the CBF was reduced to 13-18% of baseline values (Figure 1). As shown in Table 1 below, APC treatment resulted in 25% ($p = 0.05$) improvement in CBF during occlusion phase relative to control group.

TABLE 1 Effect of APC treatment on CBF during MCA occlusion and reperfusion

	Pre-occlusion (45-60 min)		Occlusion (0-60 min)		Reperfusion (0-60 min	
	Control n=7 mice	APC n=6 mice	Control n=7 mice	APC n=6 mice	Control n=7 mice	APC n=6 mice
Ischemic hemisphere	100.0±1.2	105.0±5.1	11.7±3.5	14.3±5.9*	32.3±7.9	78.6±22.5**
Contralateral hemisphere	100.0±1.4	103.0±5.6	91.9±19.3	95.7±7.8	85.8±17.2	93.0±7.9

Values are mean ± SD. The number of CBF measurements during each studied period of time was 6 to 24 for the mean values listed in Table 1. Values are expressed as a percentage of baseline pre-occlusion CBF values determined within 30 min prior to MCA occlusion in either group. *p=0.05 and **p<0005 for CBF values during occusion and reperfusion respectively.

APC, however, did not affect the blood flow in the contralateral non-ischemic hemisphere (point B). During reperfusion, the CBF in the ischemic hemisphere did not exceed 32% of baseline values (Figure 1A). In contrast, there was a remarkable improvement in CBF during reperfusion in the APC-treated mice, and the values ranged between 71-98% of baseline readings and almost approached control pre-ischemic values (Figure 1B). The relative increase in CBF during reperfusion in APC-treated animals vs. control was 2.4-fold ($p < 0.0005$, Table 1). Again, APC treatment did not affect CBF in non-ischemic hemisphere.

There was a pronounced effect of APC on survival time and neurologic scores after MCA occlusion/reperfusion. As indicated in Table 2 below, a stroke-related death (score 5) with the present technique was observed in 6 out of 7 control animals between 6 and 13 hrs of reperfusion, and only 1 animal survived 24 hrs.

TABLE 2 Survival time and motor neurologic scores at 24 hrs of reperfusion

Group	Survival Time (mean±SD)	Scores at 24 hrs No. of mice	
		0 1 2 3 4 5	Score (mean±SD)
Control	10.2±2.24	0 0 0 0 1 6	4.86±0.38
APC	23.7±0.33*	0 2 2 1 0 1	2.33±1.51**

*p<0.005 for survival by Kruskal-Wallis test
**p<0.01 for scores by Chi-square test with Fisher's transformation

The mean time of survival in control group was 10.2 hrs. In contrast, 5 out of 6 APC-treated animals survived 24 hrs, and only 1 animal died at 23 hrs. APC-treated animals were sacrificed at 24 hrs to determine the volume of brain injury, and the mean time of survival in APC-treated group was 23.7 hrs. The motor neurological score in APC-treated animals was about 2-fold lower than in control group.

Mice treated with APC had a significant reduction in the volume of brain injury compared to control mice (Figure 2). The total volume of injury of gray matter (corrected for edema), i.e., the brain infarction volume, was significantly decreased by 59% ($p < 0.02$) in the APC-treated group relative to control mice (Figure 2A). The edema volume in the lesioned ischemic hemisphere was also reduced by APC treatment by 50% ($p < 0.05$). Studies of the infarct area for each of the five coronal sections of the same brains as in Figure 2A confirmed significant reductions of injury at all brain levels in APC-treated mice (Figure 2B). Figure 3 illustrates that 100% of control mice had injury that involved significant ipsilateral portion of the hemisphere including cortex, subcortical structures and lateral striatum; ≥ 50% mice exhibited changes in the medial striatum and < 50% showed changes in the dorsomedial and ventromedial cortex. In APC-treated animals, there was a significant reduction of the injured area, and all animals developed injury only in small well-localized area in the lateral cortex, with significant reduction of injury in each region.

Immunostaining for fibrin was performed after TTC staining.

Several microvessels including small veins, arterioles and numerous capillaries contained intraluminal fibrin deposits corresponding to grade 1 according to the scale of fibrin localization in an MCA model (P. Tabrizi et al., 1999 and Y. Okada et al., 1994). Extravascular deposition of fibrin grade 2 was also found around some microvessels in control mice. The migration of leucocytes into parenchymal tissues was frequently observed in control animals, and PMNs were identified either by morphology or by positive staining for CD11b in the tissue, and/or positive staining for dichloroacetate esterase. In addition to a single antibody staining, sections were also counter-stained with hematoxylin. Double staining for fibrin and leukocytes showed complete thrombosis of a large venule. The expansion of so-called "white thrombus" from the vein into a capillary bed was also shown. The nuclear morphology of neutrophils could not be seen as hematoxylin staining was not performed simultaneously with double staining. Staining with dichloroacetate esterase confirmed infiltration of ischemic parenchymal tissue with PMNs in control mice . It is noteworthy that deposition of fibrin in control mice was much more significant in the present model than in a previous MCA model (Y. Okada et al., *Stroke.* 25:1847-1853, 1994). The difference could be related to significantly higher reductions in the CBF in the present vs. previous model, both during occlusion, i.e., 88% vs. 63%, and reperfusion, 68% vs. 30%, respectively. Double staining confirmed co-localization of fibrin and leukocytes in cerebral vessels in the ischemic hemisphere in control mice. APC treatment significantly reduced fibrin deposition, as well as vascular accumulation and parenchymal infiltration with PMNs.

The number of fibrin-positive microvessels in the ischemic hemisphere in the area of infarction was reduced by about 2.5-fold in APC-treated animals compared to control mice (Figure 4A). However, it is important to note that fibrin staining was much weaker on simultaneously stained tissue sections in APC-treated animals than in control mice, so that the number of fibrin positive vessels does not reflect accurately the amount of fibrin deposited in tissue, as indicated by the more sensitive quantitative Western blot analysis (Figure 5). The number of CD11-b positive cells in tissue and the number of dichloroacetate esterase positive neutrophils was the same in control mice suggesting that most (if not all) of CD11b positive cells could in fact be PMNs (Figure 4B). The number

of PMNs in tissue dropped by 11.2-fold in APC-treated animals (Figure 4B). Macroscopic inspection and histologic analysis indicated no intracerebral bleeding or subarachnoid hemorrhage in APC-treated animals. These results were corroborated by barely detectable hemoglobin levels in the ischemic hemisphere both in APC-treated animals and in control animals that were even below the values in the contralateral non-ischemic hemisphere (Figure 4C) confirming the absence of microbleeding in ischemic brain tissue. Hemoglobin values were particularly low in the ischemic hemisphere in control animals possibly reflecting minimal vascular entrapment of red blood cells due to brain swelling and impaired re-circulation.

Figures 5A and 5B illustrate 8.2-fold decrease in the amount of deposited fibrin in ischemic hemisphere of APC-treated vs. control mice determined by quantitative Western blot analysis.

EXAMPLE 2

The effects of administration of APC post occlusion was studied in a separate set of experiments using the methods and materials described above in Example 1 above, except that APC (2 mg/kg) was administered to mice 10 min after the MCA occlusion. Infarction volume was reduced by 69% ($p < 0.03$) (Figure 6A) and edema volume was reduced by and 61% ($p < 0.05$) (Figure 6B), cerebral blood flow was restored towards control values (Figure 6C), and brain accumulation of neutrophils was eliminated (Figure 6D). In addition, the decrease in the number of fibrin positive vessels in the ischemic hemisphere was 25%, an insignificant amount in comparison to vehicle-treated controls (Figure 6E). Thus, APC reduced volumes of brain infarction and edema in a dose-dependent fashion (Figures 6A and B) and produced a dose-dependent restoration in CBF during reperfusion (Figure 6C).

In this post-occlusion administration model, immunostaining for ICAM-1 in the ischemic hemisphere indicated that APC administration after the onset of ischemia reduced the intensity of ICAM-1-positive blood vessels, and the number of ICAM-1-positive blood vessels was reduced by 61% (Figure 7).

Table 3 below shows the cumulative results of experiments conducted with mice treated with APC (2 mg/kg) 15 min prior to or 10 min after the MCA occlusion had no significant differences in mean arterial blood pressure, PaO_2, $PaCO_2$, pH, hematocrit, head temperature, and blood glucose when compared with control animals (data not shown). APC administration did not influence CBF under basal conditions in the absence of occlusion/reperfusion.

TABLE 3 Survival and motor scores at 24 hrs post MCA occlusion/reperfusion

Group	Survival Time (hr)	Scores at 24 hours post MCAO	
		No. of mice	Mean Score (± SD)
Control	(mean ± SD)	0 1 2 3 4 5	
APC 15 min prior to MCAO	13.6 ± 3.24	0 0 0 2 2 8	4.50 ± 0.79
2 mg/kg APC 10 min after MCAO	23.7 ± 0.33*	0 2 2 1 0 1	2.33 ± 1.51**
2 mg/kg	24.0 ± 0.00*	1 3 1 0 0 0	1.25 ± 0.96*
0.5 mg/kg	24.0 ± 0.00*	0 1 1 1 0 0	2.00 ± 1.00*
0.1 mg/kg	14.3 ± 6.43^{ns}	0 0 0 1 0 2	4.33 ± 1.15^{ns}

APC or vehicle was administered 15 min prior to or 10 after stroke induction. The difference between control animals treated with vehicle prior to or after MCA occlusion (MCAO) was not significant and data for controls were pooled. * $p < 0.005$ for survival by Kruskal-Wallis test for APC-treated vs. control group; *$p<0.005$; **$p < 0.01$ for scores by Chi-squared test with Fisher's transformation test for APC-treated vs. control group; ns, non-significant.

The mean survival time for control group animals was 13.6 hours. Ten of 11 mice treated with 2 mg/kg APC either 15 min prior to occlusion or 10 min after occlusion survived 24 hrs, and 1 APC-treated animal died at 23 hours. All 3 animals treated with 0.5 mg/kg APC at 10 min after stroke induction survived 24 hours. APC-treated animals were sacrificed at 24 hrs to determine the volume of brain injury, thus data beyond 24 hours is not available.

The motor neurological scores in mice given 2 mg/kg APC 15 min prior to and 10 min after the MCA occlusion were improved by 2 to 2.7-fold compared with control group. Also, 0.5 mg/kg APC given 10 min after MCA occlusion improved neurological outcome significantly. However, the protective effect of APC was not apparent at an APC dose of 0.1 mg/kg based on survival time and neurologic function (Table 1) and on the volume of brain injury and effects on CBF (Fig. 7).

EXAMPLE 3

The effects of administration of protein S, a non-enzymatic cofactor of APC, was studied in a separate set of experiments using the methods and materials described above in Example 1. Either vehicle, protein S (2 mg/kg) alone or protein S (2 mg/kg) co-injected with a low dose of APC (0.1 mg/kg) was injected 10 minutes after the MCA occlusion. The results shown in Figures 8A and 8B indicate that the low dose of APC alone was not protective. However, co-injection of protein S (2 mg/kg) and APC (0.1 mg/kg) produced a synergistic effect, significantly reducing brain infarction and edema by 71% ($p<0.008$) and 51% ($p<0.05$), respectively, in the focal brain ischemia model.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

WHAT IS CLAIMED IS:

1. A method of protecting neuronal cells from cell death in a subject having or at risk of having a neuropathological disorder, comprising administering to the subject, a neuroprotective amount of activated protein C (APC), thereby providing neuroprotection to the subject.

2. The method of claim 1, wherein the APC is administered intravenously.

3. The method of claim 1, wherein the neuropathological disorder is selected from the group consisting of stroke, Alzheimer's disease, Huntington disease, Parkinson's disease, ischemia, epilepsy, amylotrophic lateral sclerosis, meningitis, multiple sclerosis, mental retardation and aging.

4. The method of claim 3, wherein the disorder is stroke and the APC is administered during the stroke or up to six hours before or after the stroke.

5. The method according to claim 1, wherein the method further comprises administering one or more antithrombotic factors or APC-cofactors.

6. The method according to claim 5 wherein the APC-cofactor is protein S.

7. The method according to claim 1 wherein the method further comprises administering one or more additional neuroprotective agents.

8. The method according to claim 7 wherein the additional neuroprotective agent is a N-methyl-D-aspartate (NMDA) receptor antagonist or a calcium ion channel antagonist.

9. A method for reducing inflammation in a subject having or at risk of having a neuropathological disorder, comprising administering to the subject, an anti-inflammatory effective amount of activated protein C (APC), thereby reducing neurological inflammation in the subject.

10. The method of claim 9, wherein the APC is administered intravenously.

11. The method of claim 9, wherein the neuropathological disorder is selected from the group consisting of stroke, Alzheimer's disease, Huntington disease,

Parkinson's disease, ischemia, epilepsy, amylotrophic lateral sclerosis, meningitis, multiple sclerosis, mental retardation and aging.

12. The method of claim 11, wherein the disorder is stroke and the APC is administered during the stroke, or up to six hours before or after the stroke.

13. The method of claim 12, further comprising administering one or more antithrombotic factors or APC cofactors.

14. The method of claim 13, wherein the APC cofactor is Protein S.

15. A method for reducing inflammation in a subject having or at risk of having inflammatory vascular disease comprising administering to the subject, an anti-inflammatory effective amount of activated protein C (APC), thereby reducing inflammation in the subject.

16. The method as in any of claims 1, 9 or 15, further comprising administering to the subject a therapeutically effective amount of one or more anticoagulant, anti-platelet or thrombolytic agent.

17. The method of claim 16, wherein the thrombolytic agent is selected from the group consisting of urokinase, tPA, Lys-plasminogen and streptokinase.

18. The method of claim 16, wherein the anti-platelet agent is selected from the group consisting of aspirin, dipyridamole, ticlopidine, clopidogrel, abciximab (Reopro) and any inhibitor of platelet glycoprotein IIb-IIIa.

19. The method as in any of claims 1, 9, or 15, further comprising administering to the subject a therapeutically effective amount of an antithrombotic factor or APC-cofactor.

20. The method of claim 19 wherein the APC-cofactor is protein S.

21. The method of claim 19 wherein the method further comprises administering to the subject a therapeutically effective amount of one or more anti-inflammatory agents.

FIG. 1A

CONTROL

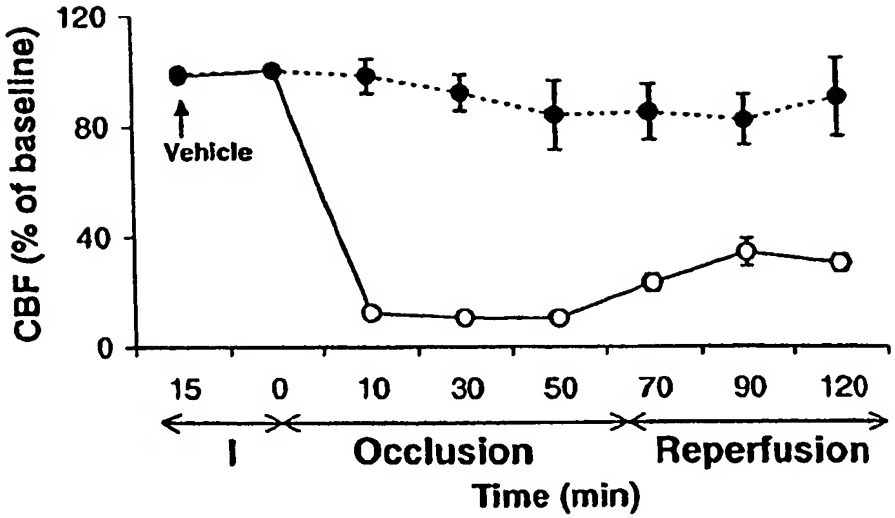


FIG. 1B

APC-TREATED

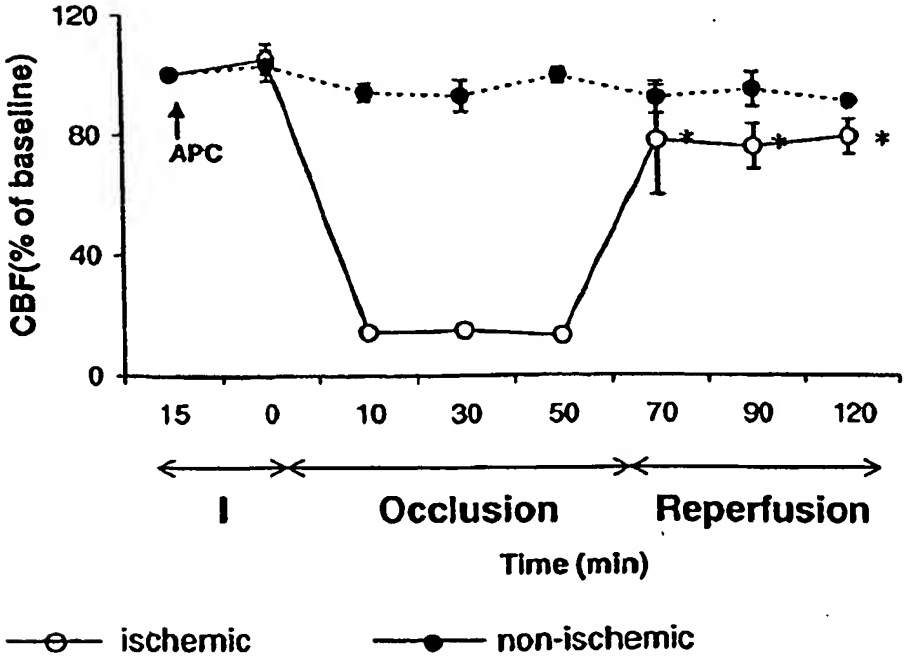
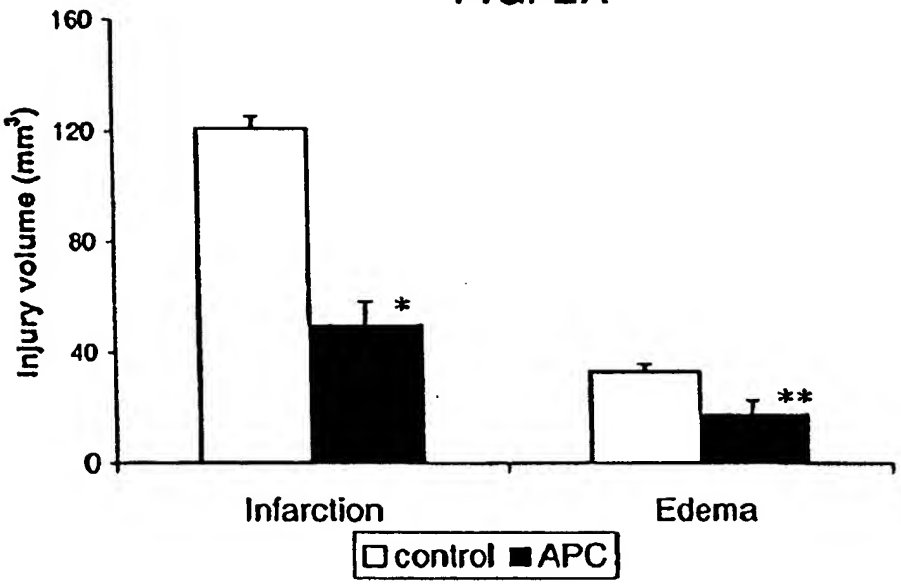


FIG. 2A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120
80
40
0
Injury volume (mm³)
*
**
Infarction
Edema
control
APC

FIG. 2B

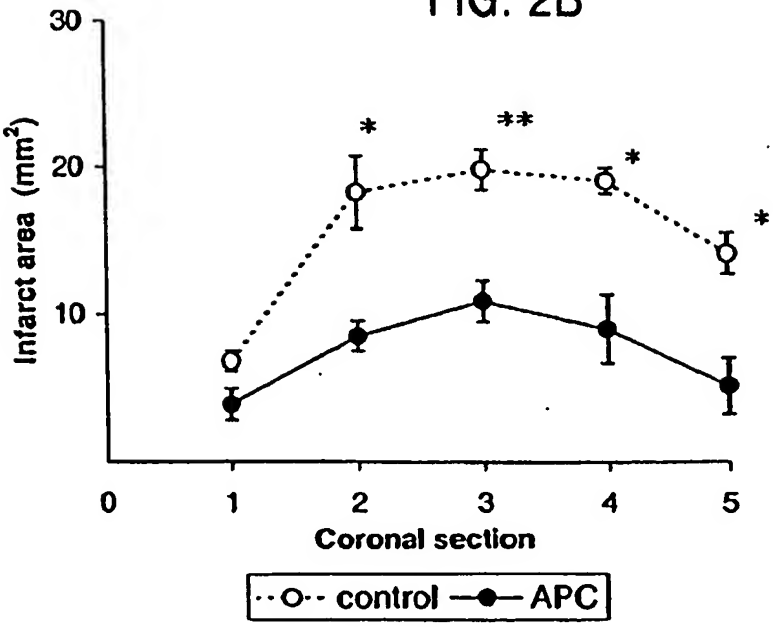

30
20
10
0
Infarct area (mm²)
*
**
*
*
1
2
3
4
5
Coronal section
control
APC

FIG. 3A
Control

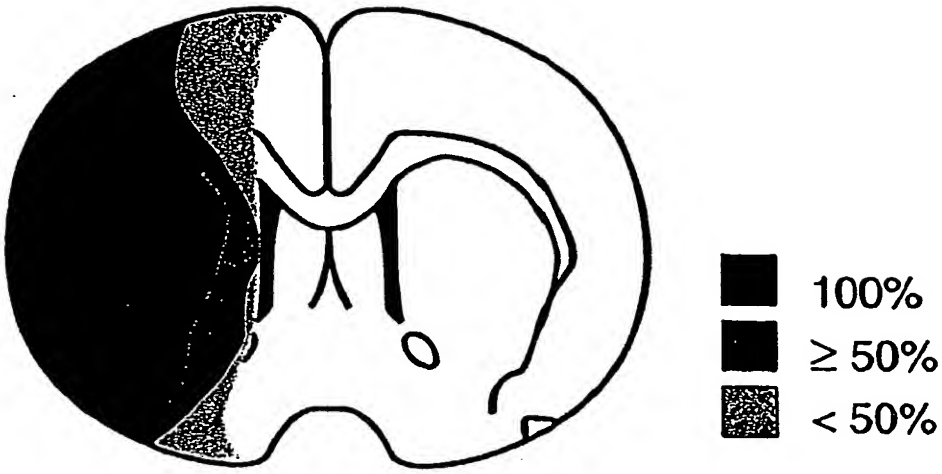


FIG. 3B
APC-Treated

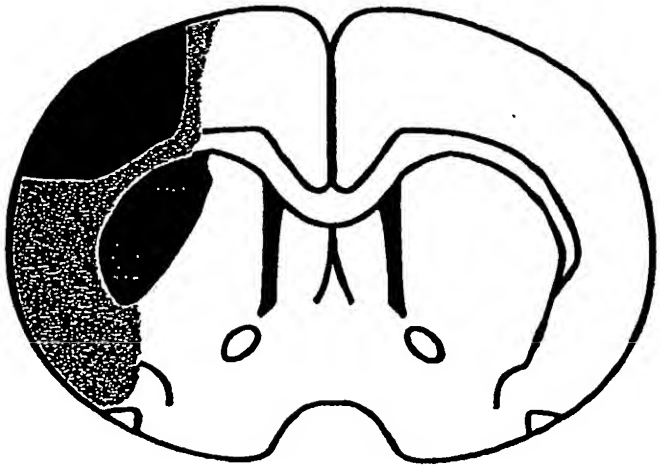


FIG. 4A

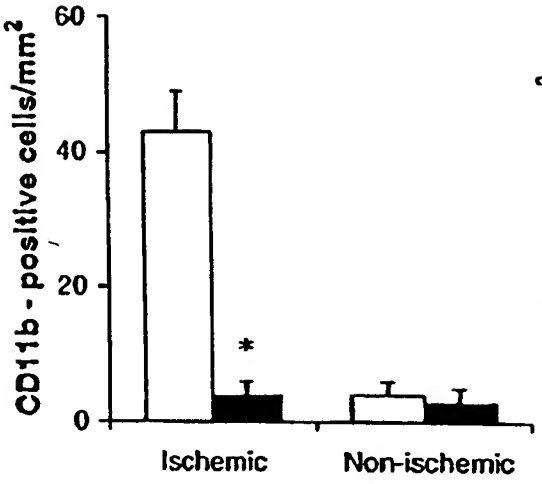


FIG. 4B

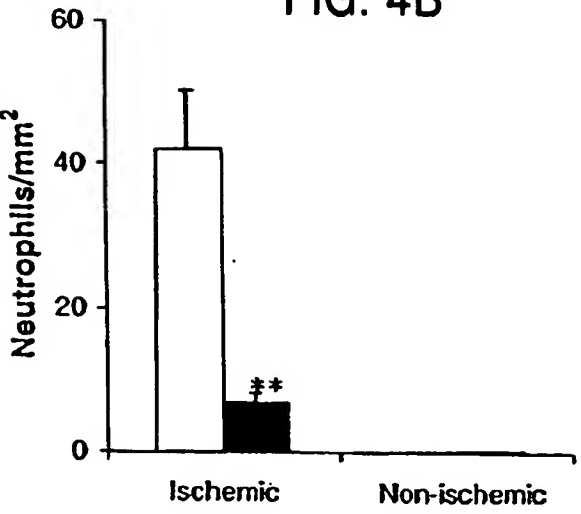


FIG. 4C

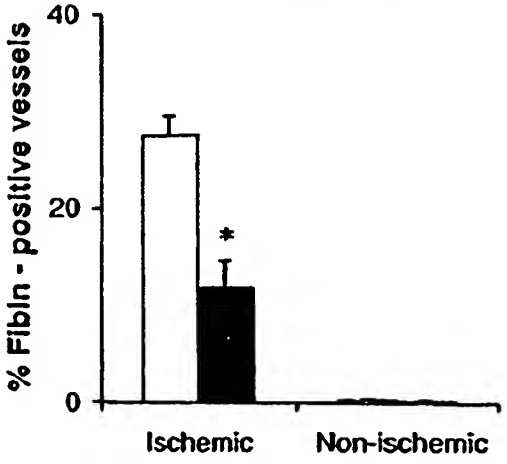


FIG. 4D

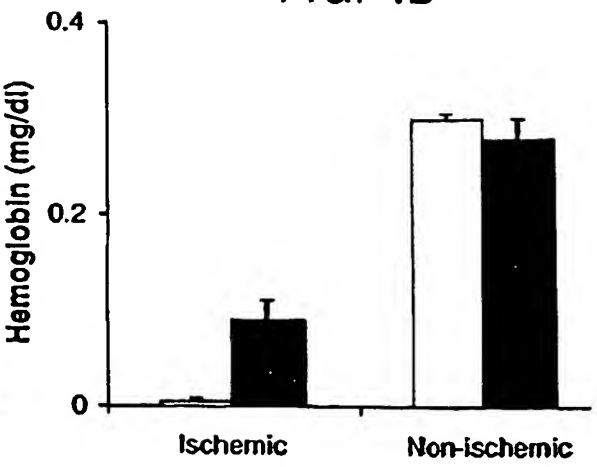


FIG. 5A

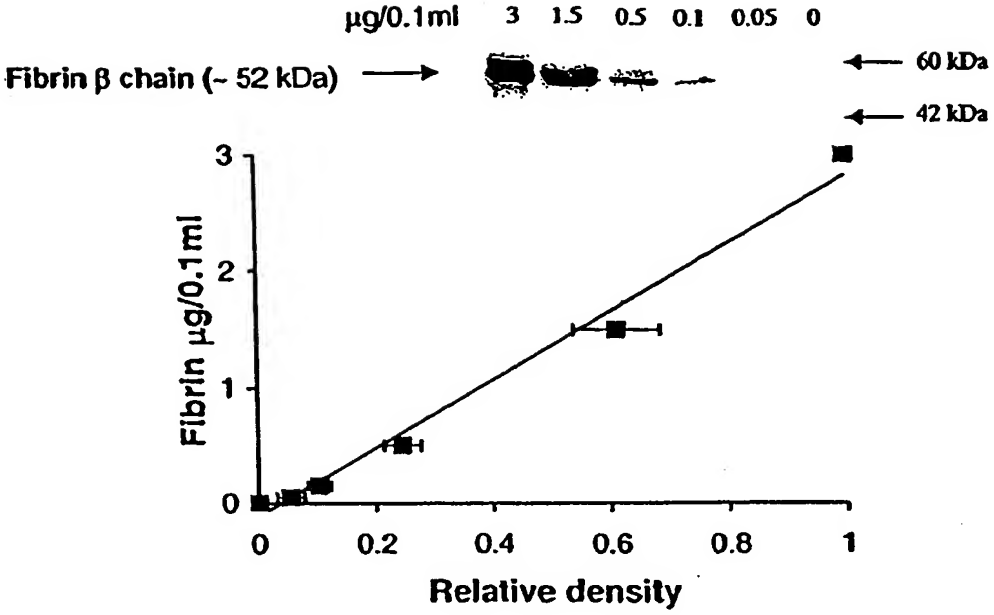
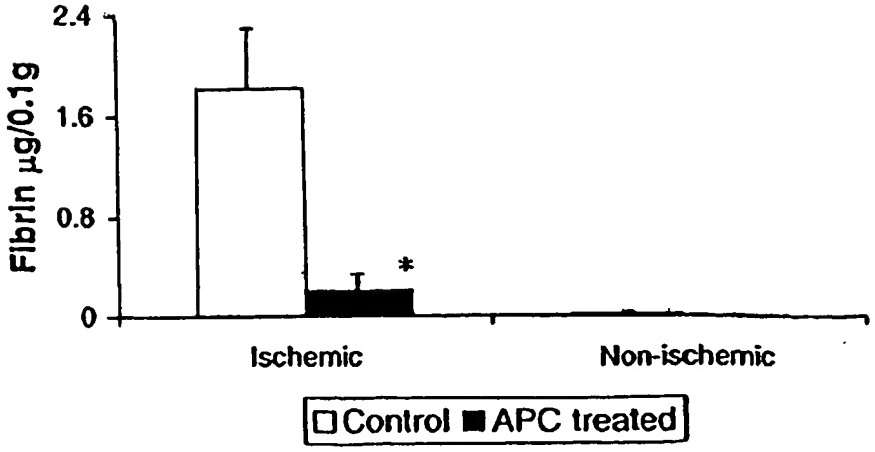

µg/0.1ml 3 1.5 0.5 0.1 0.05 0
Fibrin β chain (~ 52 kDa)
60 kDa
42 kDa
Fibrin µg/0.1ml
3
2
1
0
0 0.2 0.4 0.6 0.8 1
Relative density

FIG. 5B


Fibrin µg/0.1g
2.4
1.6
0.8
0
*
Ischemic
Non-ischemic
Control APC treated

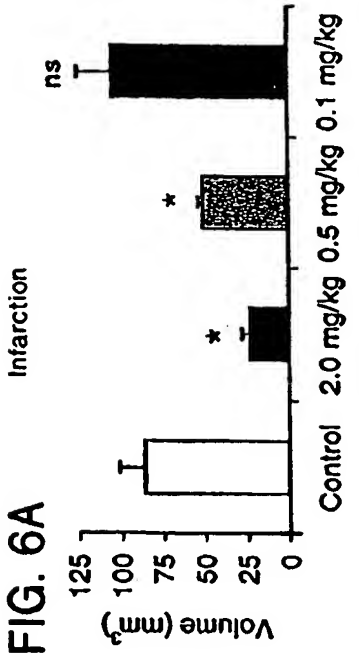


FIG. 6A

FIG. 6B

FIG. 6C

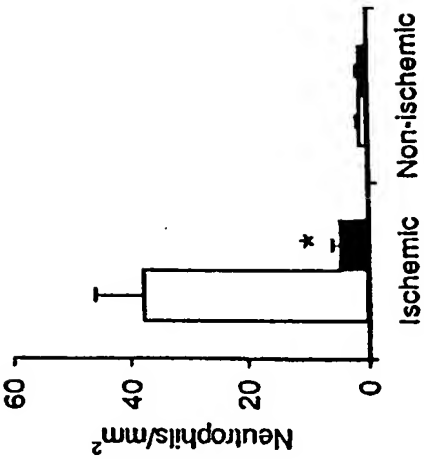


FIG. 6D

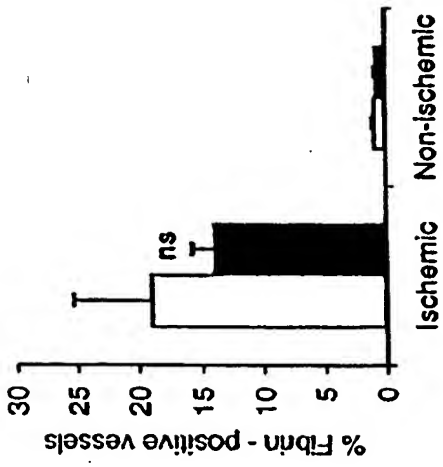


FIG. 6E

FIG. 7

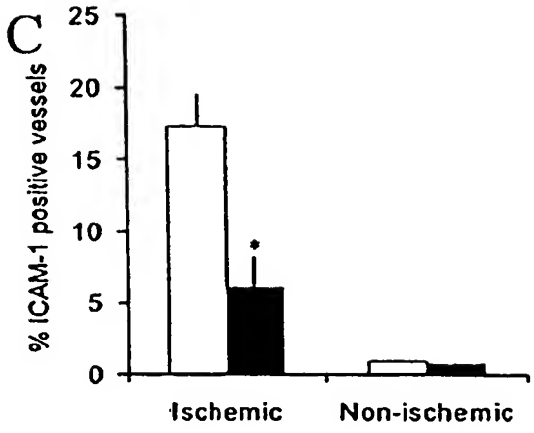

C
% ICAM-1 positive vessels
25
20
15
10
5
0
*
Ischemic
Non-ischemic

FIG. 8A

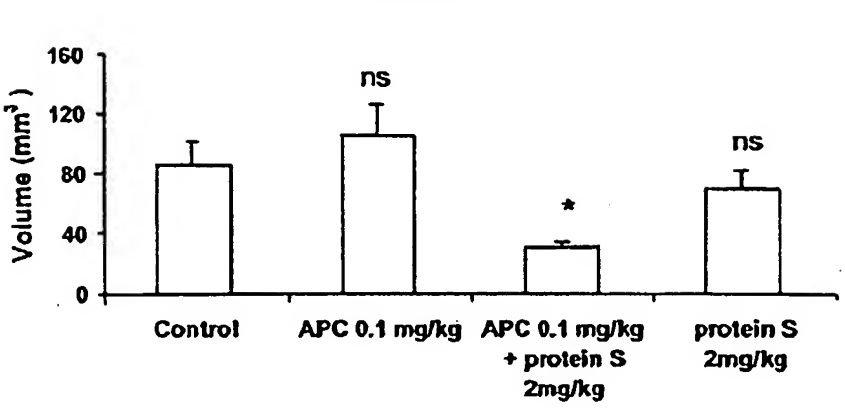
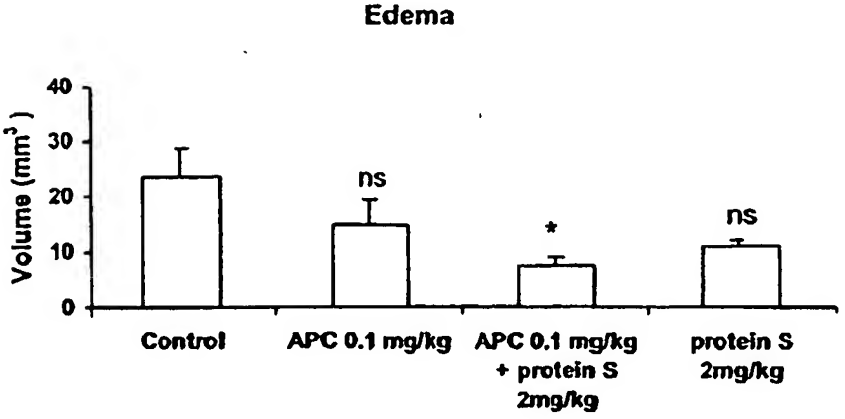

Infarction
Volume (mm³)
160
120
80
40
0
ns
*
ns
Control
APC 0.1 mg/kg
APC 0.1 mg/kg + protein S 2mg/kg
protein S 2mg/kg

FIG. 8B


Edema
Volume (mm³)
40
30
20
10
0
ns
*
ns
Control
APC 0.1 mg/kg
APC 0.1 mg/kg + protein S 2mg/kg
protein S 2mg/kg

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03758

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/00, 38/43, 39/00; C07K 2/00
US CL : 424/ 94.1, 198.1; 514/2; 530/300, 350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	SHIBATA et al. Activated Protein C Protects in a Murine Model of Ischemic Stroke. Soc. Neurosci. Abtracts. November 2000, Vol 26.No. 1-2, No. 184.19, see entire abstract.	1-4
Y	US 5,084,274 A (GRIFFIN et al) 28 January 1992 (28.01.1992), see columns 3-8.	1-5, 19
Y	MACKO et al. Brain-Specific Protein C Activation During Carotide Artery Occlusion in Humans. Stroke. March 1999, Vol 30, pages 542-545, especially pages 543-544.	1-5, 19
Y, P	US 6,037,322 A (GRINNELL et al) 14 March 2000 (14.03.2000), see entire document.	1-5, 19
A	SHIBATA et al. Anti-Inflammatory , Antithrombotic, and Neuroprotective Effects of Activated Protein C in a Murine Model of Focal Ischemic Stroke. Circulation. April 2001, Vol 103, pages 1799-1805, see entire document.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
19 June 2001 (19.06.2001)

Date of mailing of the international search report
27 JUL 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer
BRIDGET E. BUNNER

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03758

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-8 and 19-21

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/03758

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claims 1-8 and 19-21, in part, drawn to a method of protecting neuronal cells from cell death in a subject having a neuropathological disorder, comprising administering a neuroprotective amount of activated protein C.

II. Claims 9-15 and 19-21, in part, drawn to a method for reducing inflammation in a subject having or at risk of having a neuorpathological disorder, comprising adminstering an anti-inflammatory effective amount of activated protein C.

III. Claims 16-21, in part, drawn to a method of reducing inflammation in a subject having or at risk of having inflammatory vascular disease comprising administering an anti-inflammatory effective amount of protein C.

The inventions listed as Groups I, II, and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-III claim different methods. For example, Group I recites the special technical feature of protection of neuronal cells from cell death and administration of a neuroprotective agent which is not required by the methods of Groups II-III. Group II recites the special technical feature of reduction of inflammation associated with a neuropathological disorder which is not required by the methods of Groups I and III. Group III recites the special technical feature of reduction of inflammation associated with inflammatory vascular disease and administration of an anticoagulant or anti-platelet agent which is not required by the methods of Groups I-II.

Continuation of B. FIELDS SEARCHED Item 3:

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search terms: inventors' names, activated protein C, APC, stroke, co-factors, protein S, N-methyl-D-aspartate, neuro, neuronal cells

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(54) Title: PROTEIN S PROTECTS THE NERVOUS SYSTEM FROM INJURY

(57) Abstract: Protein S is a significant neuroprotectant when administered after focal ischemic stroke and prevents hypoxic/re-oxygenation injury. Purified human plasma-derived or recombinant protein S improves motor neurological function after stroke, and reduced brain infarction and edema. Protein S also enhances post-ischemic reperfusion and reduced brain fibrin and neutrophil deposition. Cortical neurons are protected from hypoxia/re-oxygenation-induced apoptosis. Thus, protein S and variants thereof are prototypes of a class of agents for preventing injury of the nervous system. In particular, a disease or other pathological condition (e.g., stroke) may be treated with such agents having one or more protein S activities (e.g., anti-thrombotic and anti-inflammatory activities, direct cellular neuronal protective effects) although the latter activities are not be required.

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PROTEIN S PROTECTS THE NERVOUS SYSTEM FROM INJURY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of provisional Appln. No. 60/414,333, filed September 30, 2002; which is incorporated by reference herein.

FEDERALLY-SPONSORED RESEARCH OR DEVELOPMENT

The U.S. Government has certain rights in this invention as provided by NIH grants HL63290 and HL21544 from the Department of Health and Human Services.

BACKGROUND OF THE INVENTION

This invention relates to the use of protein S and/or variants thereof as neuroprotective agents for treating brain disorders and other pathological conditions. The ability of protein S and variants thereof to act as cell survival factors on cells of the nervous systems are demonstrated.

Benzakour and Kanthou (*Blood* 95:2008-2014, 2000) showed that protein S is produced by smooth muscle cells derived from abdominal arteries. They suggested that protein S may be an important autocrine factor in the pathophysiology of the vasculature by acting as a mitogen for these cells. In more recent work, they induced apoptosis of abdominal vessel, smooth muscle cells using sodium nitroprusside or hydrogen peroxide. Pre-treatment with protein S reduced apoptosis and cell death (Kanthou & Benzakour in *Angiogenesis: From the Molecular to Integrative Pharmacology*, pp. 155-166, 2000). These studies did not address the role of protein S in the nervous system or, more specifically, its effects on brain endothelial cells.

The present invention addresses the need for neuroprotective compositions and methods for their use to treat diseases associated with a variety of types of nervous system damage, thrombosis, and inflammation. Because injury usually occurs after a triggering event, treatment may be initiated after such an event.

Therefore, it is an objective of the invention to show how to use protein S and variants thereof as neuroprotective agents. A long-felt need for new therapeutic and prophylactic compositions is addressed thereby. Also provided are compositions that have been formulated to deliver protein S or variants thereof to the central nervous system and processes for using and making the aforementioned products. Further objectives and advantages of the invention are described below.

SUMMARY OF THE INVENTION

The present invention is directed to improved protection of cells of the nervous system. An effective amount of protein S or at least one variant thereof may be used. It may or may not have one or more optional activities: for example, inhibition of any combination of cellular stress, apoptosis, injury, or cell death; prevention of cell injury or tissue damage caused by ischemia, hypoxia, re-oxygenation, reperfusion, or the like; and anti-thrombotic and/or anti-inflammatory activity.

The subject in need of treatment may be at risk for or already affected by the disease or other pathological condition. Treatment may be initiated before and/or after diagnosis. An indication that treatment is effective may be increased function or improved neurological outcome including improved motor neurological performance, improved performance on psychiatric tests, improved level of cognitive performance; decreased brain damage due to head injury, ischemic injury, infarction, edema, or a combination thereof; decreased injury of the nervous system; or increased cerebral blood flow. Increase or decrease may be determined by comparison to treatment without protein S or variant thereof, or to the expected effects of untreated disease or another pathological condition. Other advantages and improvements are discussed below or would be apparent from the disclosure herein.

Therefore, the invention provides a treatment for therapy or prophylaxis and the products used therein. Pharmaceutical compositions may be manufactured and assessed in accordance therewith. Further aspects of the invention

will be apparent to persons skilled in the art from the following detailed description and claims, and generalizations thereto.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Figures 1A-1B show cerebral blood flow (CBF) during middle cerebral artery (MCA) occlusion/reperfusion in control (Fig. 1A) and protein S-treated mice (Fig. 1B). Vehicle or protein S (2 mg/kg) was given 10 min after initiation of the MCA occlusion. CBF values (mean ± SD) in the ischemic (open symbols) and non-ischemic hemisphere (closed symbols) in six controls and six protein S-treated mice were measured. *$p < 0.05$ between the two groups.

Figures 2A-2D show brain injury in control and protein S-treated mice. Figs. 2A to 2C: The volumes of brain injury, infarction and edema (mean ± SE) from control mice (n = 6); mice treated with human plasma-derived protein S at 0.2 mg/kg (n = 5), 0.5 mg/kg (n = 6) or 2 mg/kg (n = 6); and mice treated with recombinant protein S^{REC} at 2 mg/kg (n = 4); *$p < 0.01$, **$p < 0.05$, #$p = 0.059$. Fig. 2D: Infarct area in the seven coronal sections from the brains of control mice and mice treated with 0.5 mg/kg protein S (mean ± SE); * $p < 0.05$. Vehicle or protein S was given 10 min after initiation of MCA occlusion.

Figures 3A-3B show the incidence and topography of the infarction at the level of the optic chiasm in control mice (Fig. 3A) and mice receiving plasma-derived protein S (Fig. 3B). Key for the incidence is given in Fig. 3A. Vehicle (n = 6) or protein S (2 mg/kg, n = 6) was given 10 min after initiation of MCA occlusion.

Figures 4A-4C show deposition of fibrin at the level of optic chiasm and CD11b-positive leukocytes in the ischemic and non-ischemic hemispheres in control mice and protein S-treated (2 mg/kg) mice. Fig. 4A: Signal on Western blot for fibrin from the standard curve was linear between 0.15 and 3 µg of fibrin β-chain/0.1 ml; 3 µg/0.1 ml was arbitrarily set as 1 unit. Fig. 4B: Western blot analysis of 10 mg brain homogenate (mean ± SE) in control (n = 3) and protein S-treated mice (n = 3). Fig. 4C: CD11b-positive leukocytes (mean ± SE) from six controls (open bars) and six protein S-treated mice (closed bars). *$p < 0.05$.

Figures 5A-5D show neuroprotective effects of protein S in cultured mouse cortical neurons subjected for 12 hr to hypoxia/aglycemia followed by 12 hr re-oxygenation. Fig. 5A: TUNEL-positive neurons (upper) and neurons showing chromatin condensation and/or nuclear fragmentation by Hoechst staining (lower) under normoxic conditions (left), hypoxia/re-oxygenation (middle) and hypoxia/re-oxygenation with protein S (500 nM, right). Fig. 5B: TUNEL-positive neurons in the absence or presence of protein S or recombinant protein S^{REC} corrected for basal values of apoptosis. Fig. 5C: Time-course for anti-apoptotic effect of protein S. Fig. 5D: Dose-response for neuroprotective effect of protein S. Hypoxia/re-oxygenation in the absence (open squares) or presence of increasing concentrations of protein S (solid squares). Mean $\pm$ SE, from 3 to 5 cultures. $^{*}p < 0.05$ and $^{**}p < 0.01$.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

Protein S is a physiologic anti-thrombotic agent that inhibits prothrombinase complex activity on endothelial cells and platelets by inhibiting coagulation factors Va and Xa. Protein S is also a cofactor for activated protein C, a serine protease that inactivates coagulation factors Va and VIIIa (1-7). The critical physiologic anti-thrombotic role of protein S is revealed by the massive thrombotic complications suffered by infants homozygous for protein S deficiency (8,9). In adults, mild heterozygous deficiencies in protein S are reported to be associated with a risk for venous and arterial thrombosis (10-13), ischemic stroke (14,15), and cerebral thrombophlebitis (16,17).

In addition to its anticoagulant activity, protein S binds to vascular cells and is a potent mitogen (18-20). A structural homolog of protein S, the growth arrest specific gene-6 (gas6), is a survival factor (21). Gas6 rescues cells from apoptosis induced by serum withdrawal (22-24). It has been suggested that both protein S and gas6 are ligands for the Tyro3/Axl family of receptor tyrosine kinases (25). But the extent to which protein S functions in vivo as a ligand for Tyro3/Axl receptors was unclear (26) until the present results were obtained.

Ischemic strokes in humans are due to thrombotic or thromboembolic vascular occlusions (27) resulting in post-ischemic neurodegenerative disorder.

Protein S had significant anti-thrombotic activity in a rabbit model of peripheral arterial thrombosis (28), but its potential for stroke therapy has not been explored. In contrast to a fibrinolytic agent, e.g., tissue plasminogen activator (tPA) which may predispose to CNS bleeding (29) and is neurotoxic (30,31), elevated levels of bovine protein S in rabbits did not cause bleeding (28). No neuroprotection was observed.

We determined whether protein S may control ischemic brain damage by protecting cells of the brain (e.g., neurons, brain endothelial cells, vascular smooth muscle cells of brain vasculature, pericytes, astrocytes, microglia, oligodendrocytes, and stems cells including neuronal or oligodendrocyte precursors) from ischemic/hypoxic injury; promoting anticoagulation; controlling cerebrovascular thrombosis; or combinations thereof.

Hereditary protein S deficiency is an autosomal dominant disorder that is associated with a risk of recurrent and inappropriate clot formation. Most likely consequences are venous thrombosis and pulmonary embolism, but protein S deficiency may also predispose patients to arterial thrombotic disease. Few homozygous or compound heterozygous subjects have been reported. Such a genotype may be incompatible with survival to adulthood without treatment because of the development of severe purpura fulminans shortly after birth.

While protein S deficiency in the general population is relatively rare (up to a few percent), it is found in up to 10% of young patients with venous thrombosis. Many other circumstances may lead to acquired protein S deficiency such as oral anticoagulant therapy, oral contraception, liver disease, nephrotic syndrome, disseminated intravascular coagulation, and pregnancy. Other factors that affect protein S activity are gender (women have a lower protein S level than men) and age (total protein S level increases with age in women due to their hormonal status). Total and free protein S levels are also positively correlated with triglyceride and cholesterol levels.

In neurodegenerative diseases, neuronal cells degenerate to bring about deterioration of cognitive function. A variety of diseases and neurological deficiencies may bring about such degeneration including Alzheimer's disease, Huntington disease or chorea, hypoxia or ischemia caused by stroke, cell death

caused by epilepsy, amyotrophic lateral sclerosis, mental retardation and the like, as well as neurodegenerative changes resulting from aging.

The neuroprotective activity of protein S and its functional variants may also be obtained by increasing its biological activity (e.g., gene therapy, gene activation), decreasing the biological activity of an inhibitor (e.g., reducing C4b binding protein amount or activity), and other methods of altering protein S activity.

The present invention is useful for treating many clinical conditions involving inflammatory processes. For example, inflammatory bowel disease including Crohn's disease and ulcerative colitis are spontaneous chronic inflammations of the gastrointestinal tract which involve activation of inflammatory cells whose products cause tissue injury. Neutrophils, eosinophils, mast cells, lymphocytes, and macrophages may contribute to the inflammatory response.

The present invention is also directed to treatment of systemic shock and many resultant clinical conditions associated therewith. Systemic shock often occurs as a complication of severe blood loss, severe localized bacterial infection, or ischemia/reperfusion trauma and it is a major cause of death in intensive care units. Many cases of septic shock are induced by endotoxins (i.e., lipopolysaccharides or LPS) from gram negative bacilli or toxins (i.e., toxic shock toxin 1) from gram-positive cocci bacteria. The release of LPS in the bloodstream causes release of inflammatory mediators (e.g., cytokines, platelet activating factor, complement, leukotrienes, oxygen metabolites, and the like) which cause myocardial dysfunction, vasodilation, hypotension, endothelial injury, leukocyte adhesion and aggregation, disseminated intravascular coagulation, adult respiratory distress syndrome (ARDS), or failure of liver, kidney, or central nervous system (CNS). Shock due to blood loss also involves inflammatory mediator release. In each case, inflammatory responses are induced at the original site of trauma, and also in the vasculature and remote vascularized sites.

Myocardial ischemia is associated with activation of the complement system which further promotes cardiac injury with the enhancement of a series of inflammatory events. Life threatening local and remote tissue damage occurs

during surgery, trauma, and stroke when major vascular beds are deprived for a time of oxygenation (ischemia) then restored with normal circulation (reperfusion). Reperfusion injury is characterized by vascular permeability leading to edema and infiltration of inflammatory cells. Neutrophils contribute significantly to reperfusion damage by generating oxidants or releasing proteases that damage the microvasculature or adjacent tissue. Cell death and tissue damage due to complement and inflammatory cell mechanisms lead to organ failure or decreased organ function. The activation of mediators by a local injury can also cause a remote injury to highly vascularized organs. The compositions and methodologies of the present invention are useful in the treatment of such injury.

Inflammatory response damage also occurs in glomerulonephritis as well as tubule disease. Infiltration of inflammatory cells (especially macrophages) is linked to proteinuria accompanied histologically by hypercellularity and crescent formation in glomeruli. Over a longer term, the infiltration of inflammatory cells is associated with accumulation of extracellular matrix and sclerosis and chronic compromise of renal function. The present invention is also efficacious in treating glomerulonephritis and tubule disease.

There are many other disease and pathological conditions which benefit from the methodologies of the present invention such as for example, coronary arterial occlusion, cardiac arrhythmias, congestive heart failure, cardiomyopathy, bronchitis, acute allergic reactions and hypersensitivity, neurotrauma, graft/transplant rejection, myocarditis, insulin dependent diabetes, and stroke. Stroke involves a very strong inflammatory response, that in part may be responsible for neuronal damage directly by allowing leukocytes to enter the extravascular regions of the brain and destroy normal brain cells and neurons, and indirectly by obstructing microvessels and stopping blood flow due to the procoagulant effects of inflammation. These intravascular and extravascular processes may require adhesion molecules and cytokines that are direct or indirect targets of cellular interactions which are independent of anticoagulant effects.

In addition to treating patients suffering from the trauma resulting from heart attack, patients suffering from actual physical trauma could be treated in order to relieve the amount of inflammation and swelling which normally result after an area of the body is subjected to severe trauma. Also, patients suffering from hemorrhagic shock could be treated to alleviate inflammation associated with restoring blood flow. Other disease states which might be treated using formulations of the invention include various types of arthritis, various chronic inflammatory conditions of the skin, insulin-dependent diabetes, and adult respiratory distress syndrome. After reading the present disclosure, those skilled in the art will recognize other disease states and/or symptoms which might be treated and/or mitigated by the present invention.

Some examples of arterial thrombosis where protein S alone or in combination with a thrombolytic agent, anticoagulant, anti-platelet agent, or anti-inflammatory agent is useful include the following clinical settings: i) acute arterial thrombotic occlusion including coronary, cerebral, or peripheral arteries; ii) thrombotic occlusion or restenosis after angioplasty; iii) reocclusion or restenosis after thrombolytic therapy; and iv) venous thrombotic occlusion. Thrombolytic agents such as t-PA salvage ischemic tissue when used within hours of acute heart attack or stroke by re-establishing blood flow in the occluded artery. Between one-fourth and one-third of patients who have successful thrombolytic reperfusion of occluded coronary arteries subsequently undergo reocclusion after discontinuing t-PA infusion. This complication occurs despite full-dose heparin therapy. The present invention may have greater efficacy than heparin in preventing reocclusion. Problems with thrombolytic therapy with t-PA include neurotoxicity and killing of neurons. The addition of protein S might reduce or prevent such unwanted consequences. v) Small and large caliber vascular graft occlusion. Vascular grafts of small caliber, i.e., 3-/mm diameter, have a high frequency of thrombotic occlusion. Protein S alone or in combination with a thrombolytic agent is useful to prevent occlusion. vi) Hemodialysis. The prosthetic surfaces and flow design of all hemodialyzers are thrombogenic. Currently heparin is infused during dialysis. However, heparin is only partially effective, thereby limiting the reuse of dialyzers. Also, heparin has a number of

troublesome side effects and complications. vii) Cardiopulmonary bypass surgery. To prevent thrombus formation in the oxygenator and pump apparatus, heparin is currently used. However, it fails to inhibit platelet activation and the resultant transient platelet dysfunction which predisposes to bleeding problems post-operatively. viii) Left ventricular cardiac assist device. This prosthetic pump is highly thrombogenic and results in life threatening thromboembolic events – complications that are only partially reduced by conventional anticoagulants (heparin or coumarin drugs). ix) Total artificial heart and left ventricular assist devices. x) Other arterial thrombosis. Protein S is useful for arterial thrombosis or thromboembolism where current therapeutic measures are either contraindicated or not effective. For example, protein S is useful for treating acute pre-or post-capillary occlusion, including transplantation, retinal thrombosis, or microthrombotic necrosis of any organ complicating infections, tumors, or coumarin treatment.

In another embodiment, the present invention provides methods for protecting cells of the nervous system from cell death in a subject having or at risk of disease or another pathological condition. The method includes administering an effective amount of protein S to the subject to provide neuroprotection. Examples of such disorders include, but are not limited to, stroke, Alzheimer's disease, Huntington disease, ischemia, epilepsy, amyotrophic lateral sclerosis, mental retardation and aging. One "having or at risk of having" an inflammatory vascular disease as described herein is a subject either exhibiting symptoms of the disease or diagnosed as being at risk for developing the disease. Such subjects include those having undergone or preparing for surgical procedures as described below.

In yet another embodiment, the invention provides methods for reducing inflammation in a subject having or at risk of having a neuropathological disorder. The method includes administering an anti-inflammatory effective amount of protein S to the subject, thereby reducing neurological inflammation in the subject. The methodologies of the present invention may also be efficacious in treating multiple sclerosis (MS) in addition to neuropathologies described above. MS is often characterized by the penetration of the blood-brain barrier by

circulating leukocytes, leading to demyelination in various parts of the brain, impaired nerve conduction and, ultimately, paralysis.

The term "neuroprotective" is used to denote protection of any type of cell of the nervous system including neurons, brain endothelial cells, brain vascular smooth muscle cells, pericytes, astrocytes, oligodendrocytes, stem cells including neuronal and oligodendrocyte precursors, and microglia from cellular stress, injury, and/or cell death, including ischemia and hypoxia.

The term "neurodegenerative disease" is used to denote conditions which result from loss of neurons, neuronal cell injury or loss, and/or injury of other types of brain cells such as oligodendrocytes or brain endothelial cells and/or other vascular cells, but not limited to any cell type in the nervous system which may bring about deterioration of motor or sensory functions of the nervous system, cognitive function, higher integrative intellectual functions, memory, vision, hearing etc. Such degeneration of neural cells may be caused by Alzheimer's disease characterized by synaptic loss and loss of neurons; Huntington disease or chorea; by pathological conditions caused by temporary lack of blood or oxygen supply to the brain, e.g., brought about by stroke; by epileptic seizures; due to chronic conditions such as amyotrophic lateral sclerosis, mental retardation; as well as due to normal degeneration due to aging. It should be noted that diseases such as stroke and Alzheimer's have both a neurodegenerative and an inflammatory vascular component and thus are treated by the methods of the invention.

One aspect of the invention includes the neuroprotective activity of protein S. The term "neuron" includes hundreds of different types of neurons, each with distinct properties. Each type of neuron produces and responds to different combinations of neurotransmitters and neurotrophic factors. Neurons are thought not to divide in the adult brain, nor do they generally survive long in vitro. The method of the invention provides for the protection from cell death or injury of neurons from virtually any region of the brain and spinal cord. Neurons include those in embryonic, fetal or adult neural tissue, including tissue from the hippocampus, cerebellum, spinal cord, cortex (e.g., motor or somatosensory cortex), striatum, basal forebrain (e.g., cholenergic neurons), ventral mesence-

phalon (e.g., cells of the substantia nigra), and the locus ceruleus (e.g., neuro-adrenaline cells of the central nervous system).

GENETICS AND STRUCTURE OF PROTEIN S

The human genome contains two genes: PROS1 is functional and encodes protein S and PROS2 is a pseudogene. Both genes are located on chromosome 3 at 3p11.1-3q11.2 and are linked within 4 cM. The PROS1 gene occupies about 80 kb of DNA with 15 exons. PROS1 and PROS2 are 97% and 95% identical between corresponding exons and introns, respectively. PROS2 lacks exon 1 and contains multiple base changes in the coding portions with termination codons at amino acid residues 61, 299, 410 and 522 a frameshift mutation in exon 10. Three mRNA species are transcribed from PROS1 and then translated into human protein S. The major mRNA species is about 4 kb. Three frequent polymorphisms have been described in the protein S gene: the first is located in the coding region (Pro 626 encoded either by CCA or by CCG) and the other two are located in noncoding regions (a C to T transition in intron 5 which is four bases downstream of exon 11, and a C to A transversion which is 520 bases downstream of the Stop codon).

The intron-exon organization of the gene for protein S reflects its modular structure. The first eight exons encode structural/functional domains also found in other vitamin K-dependent coagulation proteins (except for exon IV, coding for the thrombin-sensitive loop) and have been placed upstream of the ancestral gene of a steroid hormone binding protein. The 3' part of exon 1 codes for the signal peptide, exon 2 for the propeptide and the GLA-domain, exon 3 for the helical stack domain, exon 4 for the thrombin-sensitive loop, exons 5 to 8 for four epi-dermal growth factor-like domains, and exons 9 to 14 and the first 161 bp of exon 15 for the sex hormone-binding globulin-homologous domain. At least partial sequences for protein S from human, monkey, mouse, rat, rabbit and cow are known. After alignment, they are about 59% identical at the amino acid level.

The plasma concentration of protein S is about 25 mg/L (about 0.33 μM). The protein functions as a non-enzymatic cofactor to activated protein C (APC)

in the proteolytic degradation of factors Va and VIIIa. Protein S increases 10-fold the affinity of APC for negatively charged phospholipids. The two proteins form a putative 1:1 complex on lipid surfaces such as platelets. Protein S has a direct APC-independent anticoagulant activity by inhibition of prothrombinase activity and of factor X activating complex by binding to factor VIII. The importance of these properties in physiological anticoagulant mechanisms remains to be demonstrated. Protein S circulates in human plasma in two forms: about 40% free and about 60% bound to a regulator of the classical complement pathway, C4b-BP. Only the free protein S (about 120 nM) has cofactor activity for APC. The plasma concentration of C4b-BP is about 150 mg/L (about 0.26 μM). Interaction between protein S and C4b-BP is non-covalent and reversible. Protein S interacts with the β-chain of C4b-BP while the α-chains of C4b-BP are devoted to binding the complement protein C4b. Thus, only C4b-BP isoforms containing a β-chain (representing about 80% of circulating C4b-BP) are able to bind protein S. In the presence of calcium ions, the dissociation constant is approximately $5x10^{-10}$ M and all β-chain containing C4b-BP is linked to protein S. In healthy individuals, the concentration of free protein S is largely determined by the concentration of C4b-BPβ+ and corresponds to the molar excess of protein S over C4b-BPβ+.

Protein S in its mature form is a single-chain glycoprotein of 635 amino acids resulting from post-translational modification of a 676 amino acid precursor. It has three glycosylation sites (Asn 458, 468 and 489) and seven domains with different functional or structural roles. The signal peptide (residues -41 to -18) inserts into the rough endoplasmic reticulum and drives membrane translocation; the propeptide (residues -17 to -1) is necessary for carboxylase recognition and γ-carboxylation. These two domains are released by a cleavage reaction before secretion. The mature N-terminal part of the protein is composed of a GLA-domain (residues 1 to 37) containing 11 γ-carboxyglutamic acids which bind multiple calcium ions. The resulting stabilized structure has a high affinity for negatively-charged phospholipid membranes. The GLA-domain is followed by a short helical stack (residues 38 to 45) with a relatively high content of aromatic residues.

Whereas the above domains are present in all vitamin K-dependent proteins, the thrombin-sensitive region (residues 46 to 72) is found only in protein S. This domain contains two Cys residues (47 and 72) linked by a disulfide loop in which three peptide bonds are sensitive to thrombin proteolysis and it has recently been shown that circulating cleaved protein S is cleaved after Arg60, a site already described as sensitive to factor Xa cleavage. Whatever enzyme is responsible for the in vivo cleavage of protein S, the GLA-domain remains linked to the rest of the molecule by the disulfide bond. Since the GLA domain can no longer adopt the calcium-dependent conformation required for biological activity, protein S cannot bind to phospholipids at physiological calcium ion concentration and APC cofactor activity is lost. Therefore, this mutation may be used to separate APC cofactor activity from other activities of protein S (e.g., neuroprotective activity). These findings also suggest that the thrombin-sensitive loop interacts with APC and is involved in GLA-domain folding. Four epidermal growth factor-like domains (residues 76 to 242) are adjacent to the thrombin-sensitive region. EGF1 contains a β-hydroxylated Asp residue while the other three contain β-hydroxylated Asn. The EGF domains contain high-affinity calcium ion binding sites. The carboxy-terminal half of protein S is a large module homologous to sex hormone binding globulin. It contains two small disulfide loops formed by internal disulfide bonds. This module does not bind steroids, but contains at least two potential interaction sites with C4b-BP: residues 420 to 433 and residues 583 to 635.

Mutations in the protein S gene may be classified as qualitative or quantitative. A qualitative deficiency (type II) results in decrease protein S activity associated with normal levels of total or free protein S. Quantitative deficiencies have reductions in both total and free protein S (type I) or only free protein S (type III).

Frameshift mutations included 27 different insertions or deletions smaller than seven bases. Assay of protein S in plasma showed type I deficiency. Other quantitative defects resulted from insertions, deletions, splice site mutations, frameshift mutations, and missense mutations. Only seven different nucleotide substitutions are known to be responsible for type II deficiency. Five

are missense mutations (Arg-2Leu, Arg-1His, Lys9Glu, Thr103Asn, Lys155Glu) with three being located in the pro-peptide or the GLA domain. The plasma phenotype of the patient with the Thr103Asn mutation supports the role of this amino acid in the interaction with APC. Two splice site mutations were also associated with type II deficiency. One activated a cryptic splice site (intron g, AT, exon 8 -2) and results in a deletion of two amino acids from the protein (Ile-Asp 203-204). The other resulted in two alternative splice transcripts, lacking either exon 5 or both exons 5 and 6. An EGF1-lacking protein S species, corresponding to the exon 5-lacking transcript, was detected in the patient's plasma.

Three frequent polymorphisms and 18 rare polymorphisms do not appear to have an effect on protein S activity. They include missense changes (Pro35Leu, Arg192Lys, Thr477Met); silent changes which did not change the encoded amino acid (Leu-30Leu; Pro35Pro; Ile303Ile, Gly418Gly); and those that do not cose-gregate with the protein S deficiency (5'UT TC exon 1 -286; intron a, AG, exon 1 +7; intron a, del ATT, exon 2 -67;intron b, GA, exon 2 +5; intron g, GA, exon 8 -20, Arg49His, Thr57Ser, Met344Val, Ile518Met). Variation at one of the three glycosylation sites N-X-S/T (Ser460Pro) results in loss of the sugar modification, but no functional consequence of this change has been unequivocally demonstrated.

An electronic database is available of more than 100 different mutations and polymorphisms of human protein S (see Gandrille et al. *Thromb. Haemost.* 77:1201-1214, 1997). It is preferred that the protein S or functional variant thereof be derived from the same species as the organism being treated.

"Protein S" refers to native genes and proteins belonging to this family as well as variants thereof (e.g., mutations and polymorphisms found in nature or artificially designed). The chemical structure of the genes and proteins may be a polymer of natural or non-natural nucleotides connected by natural or non-natural covalent linkages (i.e., polynucleotide) or a polymer of natural or non-natural amino acids connected by natural or non-natural covalent linkages (i.e., poly-peptide). See Tables 1-4 of WIPO Standard ST.25 (1998) for a nonlimiting list of natural and non-natural nucleotides and amino acids. See Tables 1-4 of

WIPO Standard ST.25 (1998) for a nonlimiting list of natural and non-natural nucleotides and amino acids. Protein S genes and proteins may be recognized as belonging to this family by comparison to the human homologs PROS1 and PROS2, use of nucleic acid binding (e.g., stringent hybridization conditions of 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, at 50°C or 70°C for an oligonucleotide; 500 mM $NaHPO_4$ pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA, at 45°C or 65°C for a polynucleotide of 50 bases or longer; and appropriate washing) or protein binding (e.g., specific immunoassay under stringent binding conditions of 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.05% TWEEN 20 surfactant, 1% BSA, at room temperature and appropriate washing); or computer algorithms (Doolittle, *Of URFS and ORFS*, 1986; Gribskov & Devereux, *Sequence Analysis Primer*, 1991; and references cited therein). For example, washing may be initiated at an ionic strength, pH, and temperature equivalent to the hybridization/binding conditions (with or without blocking agents and/or surfactants), then decreasing the salt concentration or increasing the temperature with one or more changes of washing solution until the desired degree of specificity is achieved.

A "mutation" refers to one or more changes in the sequence of polynucleotides and polypeptides as compared to native protein S, and has at least one function that is more active or less active, an existing function that is changed or absent, a novel function that is not naturally present, or combinations thereof. In contrast, a "polymorphism" also refers to a difference in its sequence as compared to native protein S, but the changes do not necessarily have functional consequences. Mutations and polymorphisms can be made by genetic engineering or chemical synthesis, but the latter is preferred for non-natural nucleotides, amino acids, or linkages. Fusions of domains linked in their reading frames are another way of generating diversity in sequence or mixing-and-matching functional domains. For example, homologous protein C and protein S work best together and this indicates that their sequences may have coevolved to optimize interactions between the enzyme and its cofactor. Exon shuffling or gene shuffling techniques may be used to select desirable phenotypes in a chosen background (e.g., combining sequence changes that confer

loss of glycosylation at Asn458 and APC cofactor activity, hybrid human/mouse sequences which locate the species determinants).

Percentage identity between a pair of sequences may be calculated by the algorithm implemented in the BESTFIT computer program (Smith & Waterman. *J. Mol. Biol.* 147:195-197, 1981; Pearson, *Genomics* 11:635-650, 1991). Another algorithm that calculates sequence divergence has been adapted for rapid database searching and implemented in the BLAST computer program (Altschul et al., *Nucl. Acids Res.* 25:3389-3402, 1997). In comparison to the human sequence, the protein S polynucleotide or polypeptide may be only about 60% identical at the amino acid level (protein S from different mammals are only about 59% identical), 70% or more identical, 80% or more identical, 90% or more identical, or greater than 95% identical.

Conservative amino acid substitutions (e.g., Glu/Asp, Val/Ile, Ser/Thr, Arg/Lys, Gln/Asn) may also be considered when making comparisons because the chemical similarity of these pairs of amino acid residues are expected to result in functional equivalency in many cases. Amino acid substitutions that are expected to conserve the biological function of the polypeptide would conserve chemical attributes of the substituted amino acid residues such as hydrophobicity, hydrophilicity, sidechain charge, or size. Functional equivalency or conservation of biological function may be evaluated by methods for structural determination and bioassay.

The codons used may also be adapted for translation in a heterologous host by adopting the codon preferences of the host. This would accommodate the translational machinery of the heterologous host without a substantial change in chemical structure of the polypeptide. For example, a mammalian protein S or variant thereof may have its codons altered for translation in a bacterial or fungal host.

Protein S and variants thereof (i.e., deletion, domain shuffling or duplication, insertion, substitution, or combinations thereof) may be used to determine structure-function relationships (e.g., alanine scanning, conservative or nonconservative amino acid substitution). For example, protein S folding and processing, protein S secretion, protein S binding to phospholipids and other proteins,

any of the biological activities described herein, or combinations thereof may be related to changes in the amino acid sequence. See Wells (*Bio/Technology* 13:647-651, 1995) and U.S. Patent 5,534,617. Directed evolution by directed or random mutagenesis or gene shuffling using protein S may be used to acquire new and improved functions in accordance with selection criteria. Mutant and polymorphic variant polypeptides are encoded by suitable mutant and polymorphic variant polynucleotides. Structure-activity relationships of protein S may be studied (i.e., SAR studies) using variant polypeptides produced with an expression construct transfected in a host cell with or without expressing endogenous protein S. Thus, mutations in discrete domains of protein S may be associated with decreasing or even increasing activity in the protein's function.

FORMULATIONS AND THEIR ADMINISTRATION

Protein S or variants thereof may be used to formulate pharmaceutical compositions with one or more of the utilities disclosed herein. They may be administered in vitro to cells in culture, in vivo to cells in the body, or ex vivo to cells outside of a subject which may then be returned to the body of the same subject or another. The cells may be removed from, transplanted into, or be present in the subject.

Use of compositions which further comprise a pharmaceutically acceptable carrier and compositions which further comprise components useful for delivering the composition to a subject's brain are known in the art. Addition of such carriers and other components to the composition of the invention is well within the level of skill in this art.

A pharmaceutical composition may be administered as a formulation which is adapted for direct application to the central nervous system, or suitable for passage through the gut or blood circulation. Alternatively, pharmaceutical compositions may be added to the culture medium. In addition to active compound, such compositions may contain pharmaceutically-acceptable carriers and other ingredients known to facilitate administration and/or enhance

uptake. It may be administered in a single dose or in multiple doses which are administered at different times.

Pharmaceutical compositions may be administered by any known route. By way of example, the composition may be administered by a mucosal, pulmonary, topical, or other localized or systemic route (e.g., parenteral). "Parenteral" includes subcutaneous, intradermal, intramuscular, intravenous, intra-arterial, intrathecal, and other injection or infusion techniques, without limitation. In particular, achieving an effective amount of protein S in the central or peripheral nervous system may be desired. This may involve a depot injection into or surgical implant within the brain. Intravenous administration may be used for stroke and intra-arterial administration may be used during neurosurgery.

Suitable choices in amounts and timing of doses, formulation, and routes of administration can be made with the goals of achieving a favorable response in the subject (i.e., efficacy), and avoiding undue toxicity or other harm thereto (i.e., safety). Therefore, "effective" refers to such choices that involve routine manipulation of conditions to achieve a desired effect (e.g., neuroprotection; anti-thrombotic activity; anti-inflammatory activity; inhibition of apoptosis; or preventing the injury caused by ischemia, hypoxia, re-oxygentation, or the like).

A bolus of the formulation administered only once to a subject is a convenient dosing schedule although achieving an effective concentration of protein S in the brain may require more frequent administration. Alternatively, an effective dose may be administered every other day, once a week, or once a month. Dosage levels of active ingredients in a pharmaceutical composition can also be varied so as to achieve a transient or sustained concentration of the compound or derivative thereof in a subject and to result in the desired therapeutic response. But it is also within the skill of the art to start doses at levels lower than required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

The amount of compound administered is dependent upon factors such as, for example, bioactivity and bioavailability of the compound (e.g., half-life in the body, stability, and metabolism); chemical properties of the compound (e.g.,

molecular weight, hydrophobicity, and solubility); route and scheduling of administration; and the like. It will also be understood that the specific dose level to be achieved for any particular subject may depend on a variety of factors, including age, health, medical history, weight, combination with one or more other drugs, and severity of disease.

The term "treatment" refers to, inter alia, reducing or alleviating one or more symptoms of disease or another pathological condition in a subject. This includes therapy of an affected subject or prophylaxis of a subject at risk. For a given subject, improvement in a symptom, its worsening, regression, or progression may be determined by an objective or subjective measure. Treatment may also involve combination with other existing modes of treatment and agents (e.g., protein C, activated protein C, other anti-thrombotic agents, steroidal or nonsteroidal anti-inflammatory agents). Thus, combination treatment may be practiced.

EXAMPLES

The effects of purified human plasma-derived or recombinant protein S was examined in a murine in vivo model of focal ischemic stroke and an in vitro neuronal hypoxic/re-oxygenation injury. Protein S significantly improved motor neurological function after stroke and reduced brain infarction and edema in a dose-dependent manner. At higher concentrations protein S enhanced post-ischemic reperfusion and reduced brain fibrin and neutrophils deposition. In vitro protein S protected cultured cortical neurons from hypoxia/re-oxygenation-induced apoptosis. Protein S may be a prototype of a new class of neuroprotective agents with combined anti-thrombotic, anti-inflammatory and direct cellular neuroprotective effects to treat disease associated with ischemia, hypoxia, and other re-oxygenation injury (e.g., stroke) as well as similar diseases and other pathological conditions.

Animals. Procedures were approved by the University of Rochester's Institutional Animal Care and Use Committee. Male C57BL/6 mice (23-26 gm) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and

xylazine (10 mg/kg). Animals were allowed to breath spontaneously. Rectal temperature was maintained at 37°C ± 1°C. Their right femoral arteries were cannulated for monitoring of blood pressure and blood analysis.

Stroke model. A modification of the intravascular, middle cerebral artery (MCA) occlusion technique (33,34) was used to induce stroke. A non-siliconized non-coated 6-0 10 mm ± 1 mm long prolene suture with a rounded tip (diameter 0.20 mm) was advanced into the internal carotid artery to occlude the MCA for 1 hr followed by 23 hr of reperfusion.

Protein S, human plasma-derived (0.2, 0.5 or 2 mg/kg), human recombinant protein S (2 mg/kg) or vehicle were administered intravenously 10 min after the MCA occlusion (n = 6 per group). Protein S was purified as previously described (4).

Protein S was given at 10 min after the induction of stroke when blood flow was at a minimum to give a reasonable test of the bioactivity of protein S during ischemia in the murine model (33). In this regard, we note that the time course of pathophysiological changes in the present model is different from human strokes and the occlusion in this model is removed after one hour. Moreover, in the clinical situation in humans, spontaneous reopening of major occluded blood vessels in patients with ischemic stroke does not typically happen within one hour after the insult (27).

Cerebral blood flow (CBF) was monitored by Laser Doppler Flowmetry (LDF, Transonic Systems) (33,34). LDF probes (0.8 mm diameter) were positioned on the cortical surface 2 mm posterior to the bregma, both 3 mm and 6 mm to each side of midline. The procedure was considered successful if ≥ 80% drop in CBF was observed immediately after placement of the suture. Head temperature was monitored with a 36-gauge thermocouple probe in the temporalis muscle (Model 9000, Omega, CT).

Neurologic examinations were performed at 24 hr and scored (33): no neurologic deficit 0, failure to fully extend left forepaw 1, turning to left 2, circling to left 3, unable to walk spontaneously 4, and stroke-related death 5.

Arterial blood gases (pH, PaO_2, $PaCO_2$) were measured before and during MCA occlusion using ABL 30 Acid-Base Analyzer (Radiometer).

Unfixed 1 mm coronal brain slices were incubated in 2% TTC in phosphate buffer (pH 7.4). Serial coronal sections were displayed on a digitizing video screen (Jandel Scientific). Brain infarction and edema volume were calculated using Swanson correction (33,34).

Histopathology and fibrin detection. Leukocytes were stained using anti-CD11b antibody (DAKO) (1:250 dilution) directed against leukocyte Mac-1 (33). The number of CD11b positive cells in tissue was given per mm^2. Previous study has demonstrated that the number of CD11b positive cells and dichloracetate esterase (a specific marker for neutrophils) positive cells in the murine model of stroke is identical (33). Counting was performed in ten random fields in the ischemic hemisphere by two independent observers blinded to the specimen source or timing. Routine controls included deletion of primary antibody, deletion of secondary antibody and/or the use of an irrelevant primary antibody. The amount of fibrin was quantified in 1 mm thick brain hemisections by Western blotting using anti-fibrin II antibody (NYB-T2G1, Accurate Chemical) (1:500 dilution) as described (33,34).

Protein S ELISA Assay. The amount of human protein S in plasma of mice at 1 hr after they received protein S injections (0.2, 0.5, 2.0 or 6.0 mg/kg) was quantitated by ELISA as follows. Nunc Maxisorp microplates were coated with 20 µg/ml of polyclonal rabbit purified IgG anti-protein S (DAKO Corp.) in 0.1 M Na carbonate, pH 9.0 (150 µl/well) overnight at 10°C and then blocked with 200 µl buffer/well containing 50 mM Tris 100 mM NaCl, pH 7.4, 2% BSA for 2 hr. Aliquots (150 µl) of plasma diluted 1/400 and 1/1600 in 50 mM Tris, 100 mM NaCl, 0.02% Tween-20, 0.5 % BSA were added to wells and incubated for 2 hr. Following washing with TBS, 0.02% Tween-20, polyclonal HRP-labeled rabbit antibody (5 µg/ml, DAKO) was used with OPD substrate (Sigma Chemical) to detect bound protein S. Standard curves, valid for 5 to 125 ng/ml protein S, were made with dilutions (1/200 to 1/6400) of pooled normal human plasma

(assumed to contain 25 µg/ml protein S, George King Inc.). A plasma pool from 10 normal male mice gave no signal in this assay whereas the same plasma containing purified human protein S (final concentration of 25 µg/ml) gave a standard curve indistinguishable from pooled human plasma.

Cell Culture. Primary neuronal cortical cultures were established as described (35). In brief, cerebral cortex was dissected from fetal C56BL/6 mice at 16 days of gestation, treated with trypsin for 10 min at 37°C and dissociated by trituration. Dissociated cell suspensions were plated at 5×10^5 cells per well on 12-well corning tissue culture dishes coated with poly-D-lysine, in serum-free Neurobasal medium plus B27 supplement (GIBCO BRL). The absence of astrocytes was confirmed by negative staining for the glial fibrillary acidic protein. Cultures were maintained in a humidified 5% CO_2 incubator at 37°C for 5 days before treatment. To induce hypoxic re-oxygenation injury, five-day-old cultures were treated first for 12 hr with 95% N_2/5% CO_2 in DMEM serum-free medium without glucose, and next for 12 hr exposed to normoxic conditions and medium containing 5 mM glucose (36). Protein S, human plasma-derived (1 nM to 1,000 nM) protein S, or recombinant protein S was added to the medium throughout the entire 24 hr of the study. Cultures were next fixed for 10 min with 4% formaldehyde in PBS at 4°C and double stained with Hoechst 33258 (1 µg/ml) and TUNEL (terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling) to determine nuclear morphological changes and the number of apoptotic cells.

Oxidative Stress Model. Human microvascular brain endothelial cells (MBEC) were isolated by biopsy and cultured using methods similar to those previously reported by Mackic et al. (*J. Clin. Invest.* 102:734-743, 1998). Briefly, brain tissue was cut into small pieces, and then mechanically dissociated using a loose-fitting cell homogenizer in RPMI 1640 with 2% fetal calf serum (FCS) and penicillin/streptomycin. The homogenate was then fractionated over 15% dextran by centrifugation at 10,000 g for 10 min to obtain a brain microvessel pellet. Microvessels were further digested with 1 mg/ml of collagenase/dispase

and 5 µl/ml of DNase in FCS-enriched medium for 1 hr at 37°C. This cell suspension was centrifuged at 1000 g for 5 min, and the cell pellet was plated on fibronectin-coated flasks in RPMI 1640 with 10% FCS, 10% NuSerum, endothelial cell growth factors, nonessential amino acids, vitamins, and penicillin/streptomycin as a primary culture.

The P0 primary cultures were grown to confluence, and sorted based on LDL binding using the DiI-Ac-LDL method following the manufacturer's instructions (Biomedical Technology). Briefly, cells were incubated with DiI-Ac-LDL ligand for 4 hr at 37°C, trypsinized, and then separated by fluorescence activated cell sorting (FACS). Labeled and unlabeled human umbilical vein endothelial cells (HUVEC) were used to set gating limits as positive and negative controls, respectively. Unlabeled MBEC were used to control for possible background staining or differences based on cell size. Positively sorted cells were plated on fibronectin- or collagen-coated flasks in the medium described above. Cultures were grown in 5% CO_2 and split 1:3 at confluency with collagenase/dispase.

Subconfluent brain endothelial cell cultures (3-4 days after subculture) were treated with H_2O_2 by adding it to the culture medium for 2 hr. To induce senescence sublethal doses of H_2O_2 were determined and selected. After treatment the cells were washed with PBS (37°C) before harvesting, subculturing, or incubating with a fresh medium or 3-D collagen gels.

Statistics. Physiological variables, injury, infarction and edema volumes were compared using ANOVA followed by Dunnett's multiple comparisons test with the control group or Student's t-test when two groups were compared. Non-parametric data (neurologic outcome scores) was subjected to the Chi-squared test with Fisher's transformation.

Animals treated with protein S had no significant differences in mean arterial blood pressure, PaO_2, $PaCO_2$, pH, hematocrit, head temperature, and blood glucose when compared with control animals. Protein S administration did not influence CBF under basal conditions. During MCA occlusion, the CBF

in the control group dropped to 17-18% of baseline ($p < 0.001$); treatment with protein S (0.2 mg/kg to 2 mg/kg) did not improve the CBF during the occlusion phase (Fig. 1, Table 1). During post-ischemic reperfusion, the CBF returned to 58-52% of baseline in the control group (Fig. 1A, Table 1). Protein S at 2 mg/kg significantly improved the CBF during post-ischemic reperfusion by 21% to 26% ($p < 0.05$; Table 1), but the effects of the lower doses of protein S were either marginal or lacking (see Table 1).

Table 1: CBF after MCA occlusion (60 min) followed by reperfusion.

Treatment	Occlusion		Reperfusion	
	0-30 min	30-60 min	0-30 min	30-60 min
Vehicle	17.5 + 2.5	17.7 + 1.8	58.2 + 3.8	52.1 + 2.9
Protein S				
2.0 mg/kg	17.9 + 1.5	16.8 + 1.3	70.6 + 2.5*	65.8 +3.4*
0.5 mg/kg	16.0 + 2.1	16.9 + 3.2	65.7 + 1.6*	58.5 + 3.3
0.2 mg/kg	15.3 + 3.5	16.0 + 2.3	60.1 + 4.7	59.7 + 4.9

CBF during MCA occlusion/reperfusion in control and protein S-treated mice. Vehicle or protein S were given 10 min after initiation of MCA occlusion. CBF values (mean + SD) were averaged over studied period at time of occlusion or reperfusion and expressed as a percentage of baseline. *$p<0.05$

Control mice developed significant motor neurological deficit with a score close to 4 (Table 2). At the lowest dose (0.2 mg/kg), protein S reduced motor deficit and improved the average score by 1.4-fold, while at higher doses at 0.5 mg/kg and 2 mg/kg protein S improved significantly the motor score by 3.2-fold (Table 1).

Table 2: Motor neurological scores at 24 hr after MCA occlusion/reperfusion.

Treatment	No. of mice With score of 0 1 2 3 4 5	Score (mean ± SE)
Vehicle	0 0 0 1 5 0	3.83 ± 0.17
Plasma-derived Protein S		
2.0 mg/kg	3 1 1 0 1 0	1.17 ± 0.65*
0.5 mg/kg	3 1 1 0 1 0	1.17 ± 0.65*
0.2 mg/kg	3 1 0 0 1 0	1.00 ± 0.77*

Protein S or vehicle were administered 10 min after stroke induction. *$p<0.05$ by Kruskal-Wallis test.

Protein S-treated animals were sacrificed at 24 hr to determine the volume of brain injury. Protein S significantly reduced brain injury volume and edema volume in a dose-dependent manner by 35% and 43% at 0.2 mg/kg ($p < 0.05$), respectively, and by 59% and 62% at 2 mg/kg ($p < 0.01$), respectively (Figs. 2A and 2B). As shown for example in Fig. 2C, the infarction area was significantly reduced in four out of the seven coronal sections with 0.5 mg/kg of protein S. All control mice had significant injury in the cortex and striatum on the side of the occlusion (Fig. 3); ≥ 50% of mice exhibited changes in the medial striatum while < 50% had changes in the dorsomedial and ventromedial cortex (Fig. 3A). Protein S (2 mg/kg) limited brain injury to a small well-localized area in the striatum and spared most of the brain (Fig. 3B). Similar effects were obtained with recombinant protein S.

At 2 mg/kg, protein S reduced the amount of deposited fibrin in the ischemic hemisphere by 40% ($p < 0.05$; Figs. 4A and 4B) and the number of CD11b-positive leukocytes by 53% ($p < 0.01$; Fig. 4C).

To quantitate the circulating protein S levels in these studies, four mice were each injected with each dose of protein S. Blood samples were obtained an hour later and levels of human protein S in mouse plasma were determined using an ELISA. The mean levels of circulating human protein S were 4.9, 11.0, 51.8 or 155 μg/ml for injections of 0.2, 0.5, 2.0 or 6.0 mg/kg, respectively.

The direct effects of protein S in vitro on cultured mouse cortical neurons were studied. Neurons cultured under normoxic conditions exhibited occasionally TUNEL-positive staining and chromatin condensation (Fig. 5A, left panels). In contrast, during ischemic hypoxia/re-oxygenation injury, most of the cultured neurons were TUNEL-positive and exhibited nuclear condensation and/or fragmentation (Fig. 5B, middle panels). In the presence of protein S there was approximately 70% reduction ($p < 0.05$) in the number of apoptotic cells (Fig. 5A, right panels and Fig. 5B). Under the present experimental conditions, protection of neurons from apoptotic death by protein S was time-dependent and dose-dependent with the half-maximal effect EC_{50} at 75 nM (Figs. 5C-5D).

Neuroprotective effects of plasma-derived human protein S on primary human microvascular brain endothelial cells (MBEC) obtained from biopsies and exposed to 500 μM H_2O_2 for 1.5 hr. In this oxidative stress model, about 50-70% of cells became apoptotic as shown by TUNEL and Hoechst staining. Protein S administered two hours prior to oxidative stress reduced the number of TUNEL-positive cells in a dose-dependent manner with an EC_{50} of 200 nM. Next, it was demonstrated that IgG anti-annexin II (2.5 μg/ml) and IgG anti-Tyro3 (2.0 μg/ml) inhibit by > 95% the anti-apoptotic effects of protein S. These findings indicate that protein S acts as a cell survival factor in brain endothelium exposed to oxidative damage, and that annexin II and Tyro3 are required for its anti-apoptotic effects.

Data presented have demonstrated neuroprotective, anti-thrombotic, and anti-inflammatory effects of protein S in a murine in vivo model of focal ischemic stroke with reperfusion and direct neuronal protective effects of protein S in an in vitro model of ischemia using murine cultured cortical neurons challenged by hypoxia/aglycemia followed by re-oxygenation. In the stroke model, protein S reduced the motor neurological deficit, the infarction volume

and the edema volume in a dose-dependent manner. The effect of protein S on post-ischemic CBF during reperfusion was significant with 2 mg/kg, but marginal with an intermediate dose (0.5 mg/kg) and/or absent with a low 0.2 mg/kg dose. It is noteworthy that the same low dose of protein S (0.2 mg/kg) significantly reduced the injury and edema volumes by 35% and 43%, respectively, in spite of the lack of an observable effect on CBF.

We studied protein S in a murine in vivo model of stroke and an in vitro model of neuronal hypoxic/re-oxygenation injury. Animals received purified human plasma-derived protein S or vehicle intravenously 10 min after initiation of middle cerebral artery occlusion followed by reperfusion. Protein S at 0.2 to 2 mg/kg significantly improved the motor neurological deficit by 1.4- to 3.2-fold and reduced infarction volume by 35 to 59% and brain edema by 45 to 62% in a dose-dependent manner. Protein S at 2 mg/kg improved the post-ischemic cerebral blood flow by 21% to 26% and reduced brain fibrin deposition and infiltration with neutrophils by 40% and 53%, respectively. Intracerebral bleeding was not observed with protein S. Protein S protected cultured neurons from hypoxia/re-oxygenation-induced apoptosis in a dose-dependent manner. Recombinant human protein S exerted similar protective effects from hypoxia-induced damage as the plasma-derived protein S both in vivo and in vitro.

Significant obstructions in CBF in focal stroke might result from microvascular occlusions due to fibrin deposition, vascular accumulation of neutrophils and brain swelling (33,34). Previous studies reported significant anticoagulant activity of protein S in vitro and in vivo (2-7,28). The present study confirmed reduced fibrin deposition and reduced infiltration of brain tissue with leukocytes in the presence of protein S. Protein S alleviated ischemic cerebral coagulopathy and reduced ischemic microvascular obstructions with blood cells, thereby limiting the development of brain thrombosis and contributing to the restoration of post-ischemic brain perfusion. However, the cerebroprotective effects were also observed with the lower doses of protein S, which apparently did not affect and/or improve significantly the post-ischemic CBF. Therefore, in addition to anti-thrombotic mechanisms of protein S during

brain ischemia, we also considered other possible mechanisms including direct neuroprotective cellular effects.

Remarkably, our studies also demonstrated that protein S directly protects ischemic cultured neurons exposed to hypoxic/re-oxygenation injury in vitro. In the presence of protein S the number of cultured neurons that were TUNEL-positive and exhibiting nuclear shrinkage, chromatin condensation and nuclear fragmentation were significantly reduced in a dose-dependent manner. Under these conditions, protein S was able to spare about 70% of neurons with an EC_{50} of 75 nM. Cell binding and mitogenic effects of protein S have been demonstrated in vascular smooth muscle cells (18-20), while the anti-apoptotic effects of gas6, a structural homolog of protein S, are well established (22-24). The molecular nature of a protein S receptor as a transmembrane tyrosine kinase receptor has been debated (25,26). Direct anti-apoptotic activity of protein S has not been previously described. Some studies have suggested that both gas6 and protein S are ligands for the Tyro3/Axl family of receptor tyrosine kinases (25). However, the role of Tyro3/Axl receptors has not been confirmed by others (20,26) and the nature of the receptor for protein S on neuronal cells was unknown. It has been also suggested that mitogen activated protein kinases (MAPK) $p42/p44^{MAPK}$ mediate protein S membrane to nuclear signaling that might be involved in cell proliferation in vascular smooth muscle cells (20). Similar intracellular signaling may mediate the cytoprotective and/or neuroprotective effects of protein S.

Bleeding and intracerebral hemorrhage are potential life-threatening complications with anti-thrombotic therapy for stroke including thrombolytic treatment with tPA (29) or anticoagulant treatment with heparin (27). In addition, tPA is directly toxic for brain cells (30,31) in contrast to cellular neuroprotection conferred by protein S. This study indicated that protein S does not adversely affect hemostatic function or produce intracerebral hemorrhage, consistent with previous studies demonstrating that administration of protein S does not cause bleeding (28). Thus, protein S and variants thereof serve as prototypes of a new class of agents for clinical stroke with combined systemic

anti-thrombotic and anti-inflammatory activities as well as direct protective effects on neurons during cerebral ischemia.

REFERENCES

1. Griffin Chapter 113. Regulation of Coagulation. In: *William's Hematology 6th Edition* pp. 1435-1449 (2000).
2. Heeb et al. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J. Biol. Chem.* 268:2872-2877 (1993).
3. Hackeng et al. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. *J Biol Chem* 269:21051-21058 (1994).
4. Heeb et al. Protein S binds to and inhibits factor Xa. *Proc. Natl. Acad. Sci. USA* 91:2728-2732 (1994).
5. Koedam et al. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. *J. Clin. Invest.* 82:1236-1243 (1988).
6. Rosing et al. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. *J. Biol. Chem.* 270:27852-27858 (1995).
7. Lu et al. Comparison of activated protein C/protein S-mediated inactivation of human factor VIII and factor V. *Blood* 87:4708-4717 (1996).
8. Mahasandena et al. Clinical and laboratory observations: homozygous protein S deficiency in an infant with purpora fulmainans. *J. Pediatrics* 117:750-753 (1990).
9. Pegelow et al. Severe protein S deficiency in a newborn. *Pediatrics* 89:674-676 (1992).
10. Schwarz et al. Plasma protein S deficiency in familial thrombotic disease. *Blood* 64:1297-1300 (1984).
11. Comp et al. Familial protein S deficiency is associated with recurrent thrombosis. *J. Clin. Invest.* 74:2082-2088 (1984).

12. Comp & Esmon. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. *New Engl. J. Med.* 311:1525-1528 (1984).
13. Gladson et al. The frequency of type 1 heterozygous protein S and protein C deficiency in 141 unrelated young patients with venous thrombosis. *Thromb. Haemost.* 59:18-22 (1988).
14. Green et al. Protein S deficiency in middle aged women with stroke. *Neurology* 42:1029-1033 (1992).
15. Koller et al. Deficiency of both protein C and protein S in a family with ischemic strokes in young adults. *Neurology* 44:1238-1240 (1994).
16. Prats et al. Superior sagital sinus thrombosis in a child with protein S deficiency. *Neurology* 42:2303-2305 (1992).
17. Bostroem et al. Thrombophlébite cérébrale, phlébite surale, embolies pulmonaires et déficits en protéine S. *Rev. Neurol.* (Paris) 152:755-758 (1996).
18. Gasic et al. Coagulation factors X, Xa and protein S, as potent mitogens of cultured aortic smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 89:2317-2324 (1992).
19. Benzakour et al. Evidence for a protein S receptor(s) on human vascular smooth muscle cells. Analysis of the binding characteristics and mitogenic properties of protein S on human vascular smooth muscle cells. *Biochem. J.* 308:481-485 (1995).
20. Kanthou & Benzakour. Cellular effects and signaling pathways activated by the anti-coagulant factor, protein S, in vascular cells. Protein S cellular effects. In: *Angiogenesis: From the Molecular to Integrative Pharmacology* pp. 155-166 (2000).
21. Schneider et al. Genes specifically expressed at growth arrest of mammalian cells. *Cell* 54:787-793 (1988).
22. Bellosta et al. Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. *Oncogene* 15:2387-2397 (1997).

23. Goruppi et al. Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH-3T3 fibroblasts. *Oncogene* 12:471-480 (1996).

24. Nakano et al. Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, gas6. *FEBS Lett.* 387:78-80 (1996).

25. Stitt et al. The anticoagulation factor protein S and its relative, gas6, are ligands for the tyro 3/axl family of receptor tyrosine kinases. *Cell* 80:661-670 (1995).

26. Godowski et al. Reevaluation of the roles of protein S and gas6 as ligands for the receptor tyrosine kinase rse/tyro 3. *Cell* 82:355-358 (1995).

27. Zivin. Factors determining the therapeutic window for stroke. *Neurology* 50:599-603 (1998).

28. Arnljots & Dahlback. Antithrombotic effects of activated protein C and protein S in a rabbit model of microarterial thrombosis. *Arterioscler. Thromb. Vasc. Biol.* 15:937-941 (1995).

29. del Zoppo et al. Hemorrhagic transformation following tissue plasminogen activator in experimental cerebral infarction. *Stroke* 21:596-601 (1990).

30. Wang et al. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nature Med.* 2:228-231 (1998).

31. Flavin et al. Microglial tissue plasminogen activator (tPA) triggers neuronal apoptosis *in vitro*. *Glia* 29:347-354 (2000).

32. Goodnight & Griffin. Chapter 127. Hereditary Thrombophilia. In: *William's Hematology 6th Edition* pp. 1697-1714 (2000).

33. Shibata et al. Anti-inflammatory, antithrombotic, and neuroprotective effects of activated protein C in a murine model of focal ischemic stroke. *Circulation* 103:1799-805 (2001).

34. Tabrizi et al. Tissue plasminogen activator (tPA) deficiency exacerbates cerebrovascular fibrin deposition and brain injury in a murine stroke model. *Arterioscler. Thromb. Vasc. Biol.* 19:2801-2806 (1999).

35. Xiang et al. Evidence for p53-mediated modulation of neuronal viability. *J. Neuro. Sci.* 16:6753-6756 (1996).
36. Koretz et al. Pre- and post-synaptic modulators of excitatory neurotransmission: comparative effects of hypoxia/hypoglycemia in cortical cultures. *Brain Res.* 643:334-337 (1994).
37. del Zoppo. Microvascular response to cerebral ischemia/inflammation. *Ann. NY Acad. Sci.* 823:132-147 (1997).

Patents, patent applications, books, and other publications cited herein are incorporated by reference in their entirety.

All modifications and substitutions that come within the meaning of the claims and the range of their legal equivalents are to be embraced within their scope. A claim using the transition "comprising" allows the inclusion of other elements to be within the scope of the claim; the invention is also described by such claims using the transitional phrase "consisting essentially of" (i.e., allowing the inclusion of other elements to be within the scope of the claim if they do not materially affect operation of the invention) and the transition "consisting" (i.e., allowing only the elements listed in the claim other than impurities or inconsequential activities which are ordinarily associated with the invention) instead of the "comprising" term. Any of these transitions can be used to claim the invention.

It should be understood that an element described in this specification should not be construed as a limitation of the claimed invention unless it is explicitly recited in the claims. For example, functional variants of protein S are known as homologs, mutations, and polymorphisms in the human nucleotide and amino acid sequences. In addition, Gas6 analogs and/or receptor agonists (e.g., ligands) of annexin II or members of the Tyro3/Axl family may be used as functional equivalents of protein S and its functional variants. Thus, the granted claims are the basis for determining the scope of legal protection instead of a limitation from the specification which is read into the claims. In contradistinction, the prior art is explicitly excluded from the invention to the extent of specific embodiments that would anticipate the claimed invention or destroy novelty.

Moreover, no particular relationship between or among limitations of a claim is intended unless such relationship is explicitly recited in the claim (e.g., the arrangement of components in a product claim or order of steps in a method claim is not a limitation of the claim unless explicitly stated to be so). All possible combinations and permutations of individual elements disclosed herein are considered to be aspects of the invention. Similarly, generalizations of the invention's description are considered to be part of the invention. For example, neuroprotection may be manifested by inhibition of apoptosis, promotion of cell survival, prevention of neuronal injury and/or cell death, and other general cytoprotective effects.

From the foregoing, it would be apparent to a person of skill in this art that the invention can be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments should be considered only as illustrative, not restrictive, because the scope of the legal protection provided for the invention will be indicated by the appended claims rather than by this specification.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition which is comprised of protein S and/or at least one functional variant thereof, wherein the protein S or the functional variant is present in an amount sufficient to provide neuroprotection.

2. The composition of Claim 1, wherein protection against ischemia, hypoxia, re-oxygenation injury, or a combination thereof is provided in the nervous system of a subject in need of treatment.

3. The composition of any one of Claims 1-2, wherein inhibition of apoptosis and/or promotion of cell survival is provided in the nervous system of a subject in need of treatment, while antithrombotic effects are minimized.

4. The composition of any one of Claims 1-3, wherein the composition is adapted to protect one or more cell types in a subject's nervous system.

5. The composition of any one of Claims 1-4, wherein the protein S or the functional variant acts through one or more receptors selected from the group consisting of annexin II and Tyro3/Axl receptor tyrosine kinases.

6. A method of protecting one or more cell types of a subject's nervous system comprising administration of an effective amount of protein S and/or at least one functional variant thereof to the one or more cell types to provide neuroprotection.

7. The method of Claim 6, wherein the protein S or the functional variant is a human protein S or functional variant.

8. The method of any one of Claims 6-7, wherein the protein S or the functional variant has at least anti-thrombotic activity.

9. The method of any one of Claims 6-8, wherein the protein S or the functional variant has at least anti-inflammatory activity.

10. The method of any one of Claims 6-9, wherein the protein S or the functional variant at least inhibits apoptosis or acts as a cell survival factor.

11. The method of any one of Claims 6-10, wherein the protein S or the functional variant acts through one or more receptors selected from the group consisting of annexin II and Tyro3/Axl receptor tyrosine kinases.

12. The method of any one of Claims 6-11, wherein no protein C or activated protein C is administered.

13. The method of any one of Claims 6-12, wherein there is no deficiency of protein S activity in the subject.

14. The method of any one of Claims 6-13, wherein the protein S or the functional variant is administered to the subject after injury caused by at least ischemia, hypoxia, re-oxygenation injury, or a combination thereof.

15. The method of any one of Claims 6-13, wherein the protein S or the functional variant is administered to the subject at risk for injury caused by at least ischemia, hypoxia, re-oxygenation injury, or a combination thereof.

16. The method of any one of Claims 6-13, wherein the protein S or the functional variant is administered before and/or after diagnosis of disease or another pathological condition.

17. The method of any one of Claims 6-13, wherein cerebral blood flow in the subject's brain is increased by administration of the protein S or the functional variant.

18. The method of any one of Claims 6-13, wherein volume of the subject's brain which is affected by injury, infarction, edema, or a combination thereof is decreased by administration of the protein S or the functional variant.

19. Use of protein S or at least one functional variant thereof in an amount effective to protect against at least ischemia, hypoxia, re-oxygenation injury, or a combination thereof for the manufacture of a pharmaceutical composition.

20. Use of protein S or at least one functional variant thereof in an amount effective to at least inhibit apoptosis or act as a cell survival factor.

21. Use of protein S or at least one functional variant thereof for the manufacture of a pharmaceutical composition to at least inhibit apoptosis or act as a cell survival factor.

22. A process of screening for an agent which inhibits apoptosis and/or acts as a cell survival factor comprising:

(a) providing a library of candidate agents which are variants of protein S and

(b) selecting at least one agent by its ability to inhibit apoptosis and/or act as a cell survival factor.

23. A process of producing an agent which inhibits apoptosis and/or acts as a cell survival factor comprising:

(a) providing a library of candidate agents which are variants of protein S,

(b) selecting at least one agent by its ability to inhibit apoptosis and/or act as a cell survival factor, and

(c) producing the at least one agent.

24. An agent selected by the process of Claim 22 and/or produced by the process of Claim 23.

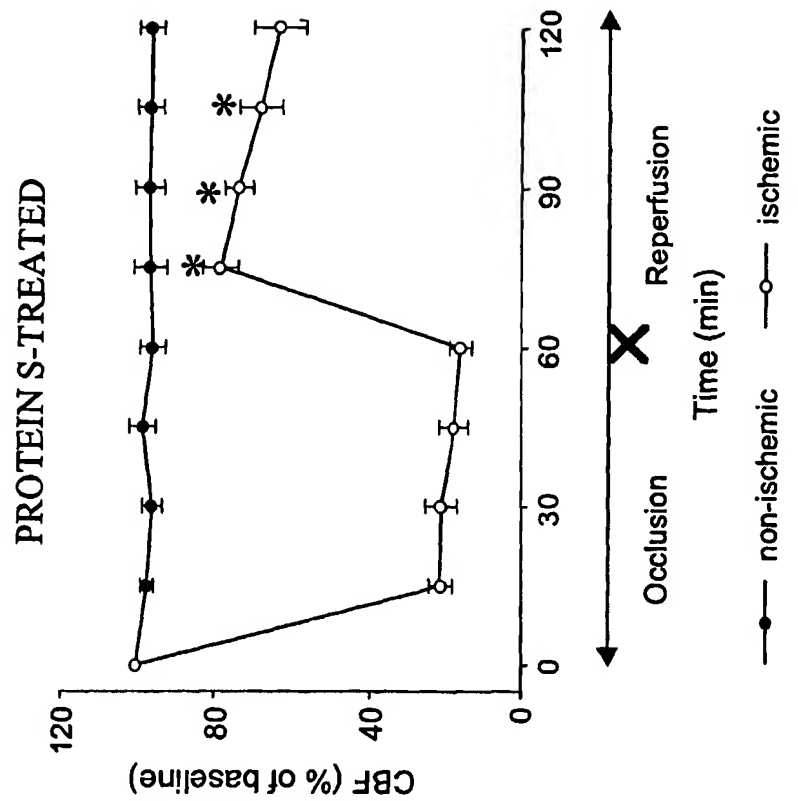


Figure 1B

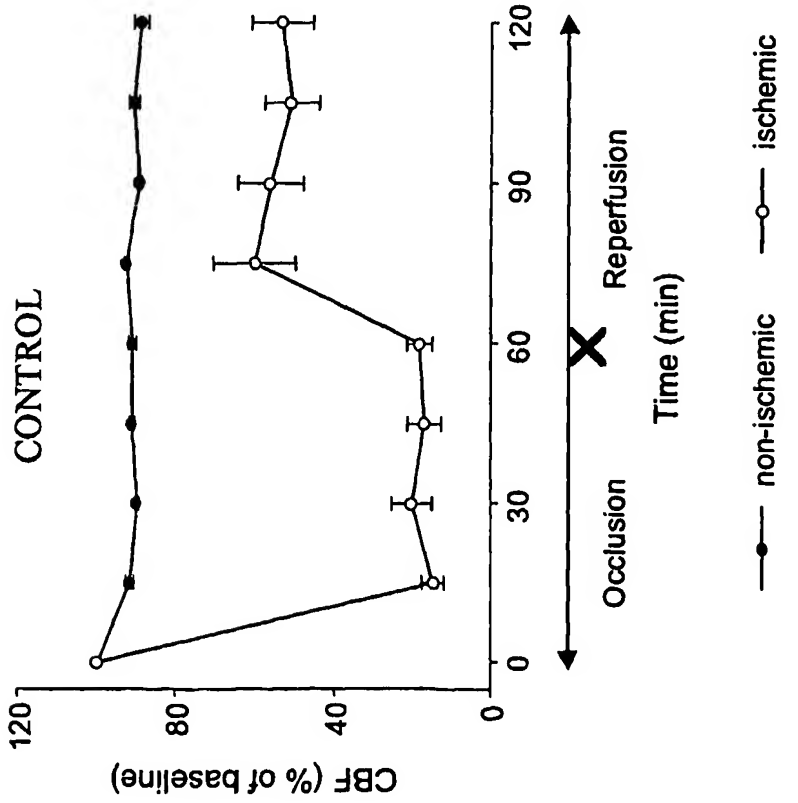


Figure 1A

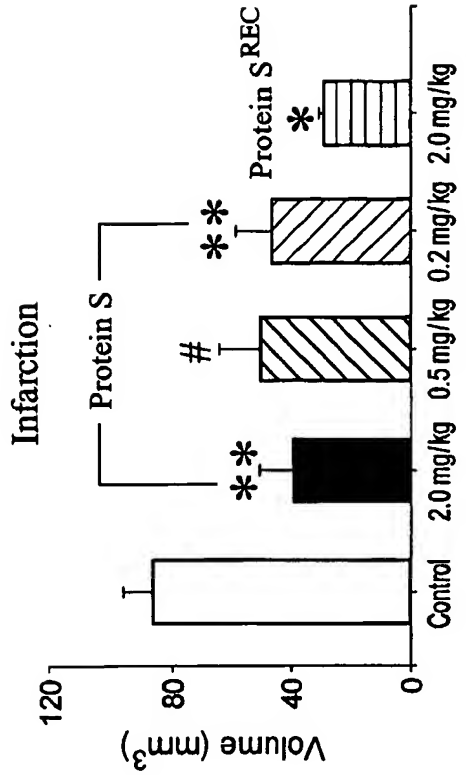


Figure 2B

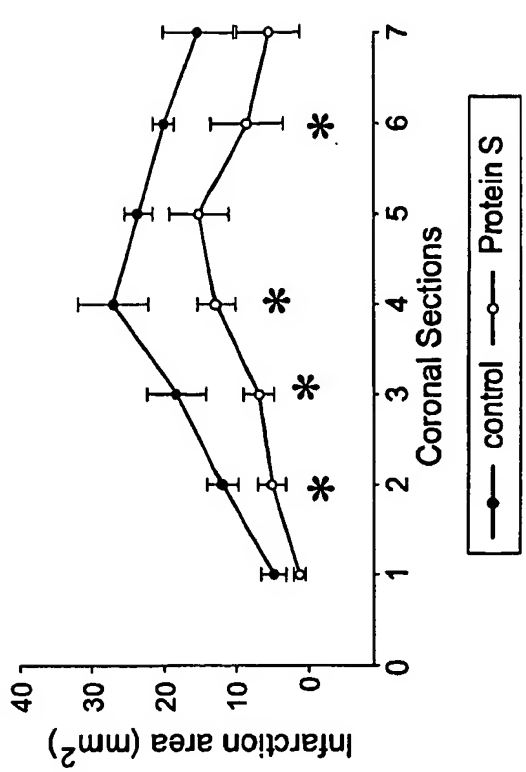


Figure 2D

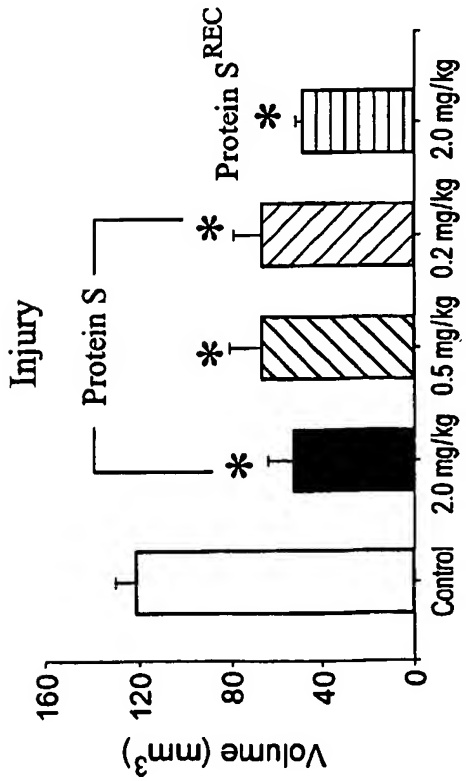


Figure 2A

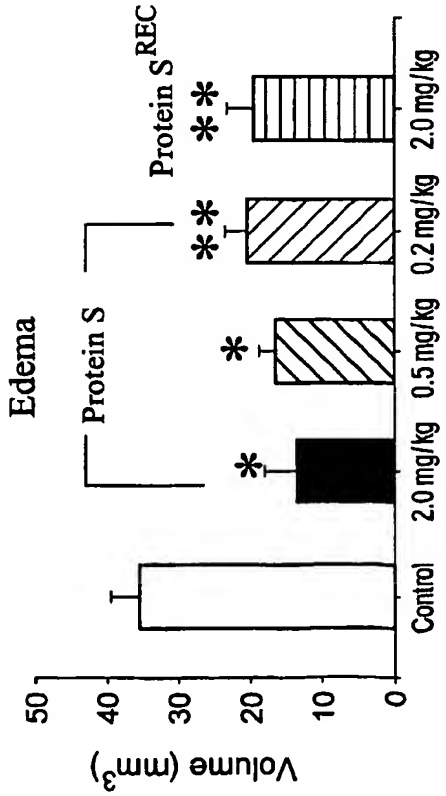


Figure 2C

Figure 3A

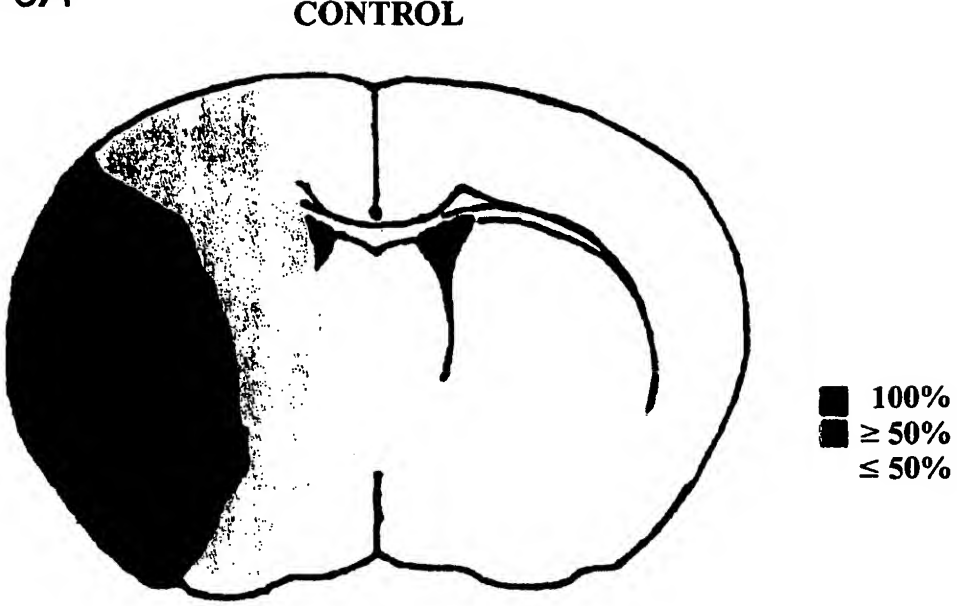


Figure 3B

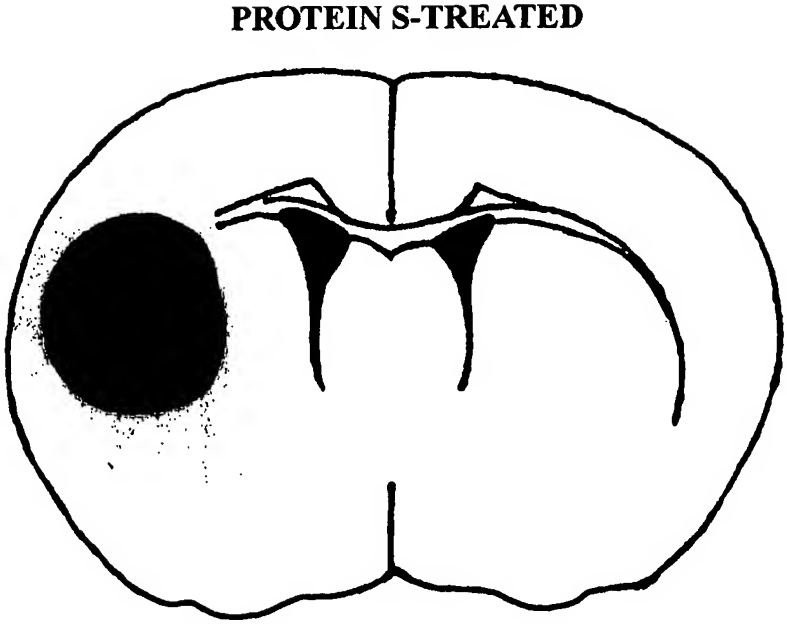
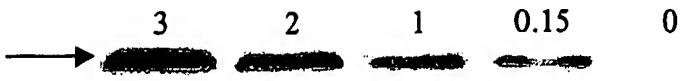


Figure 4A



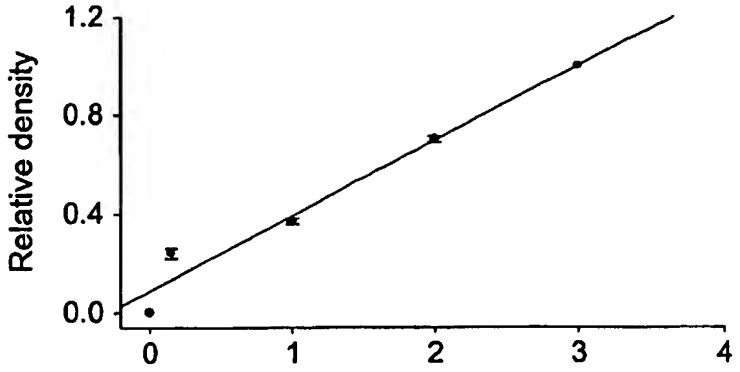


Figure 4B

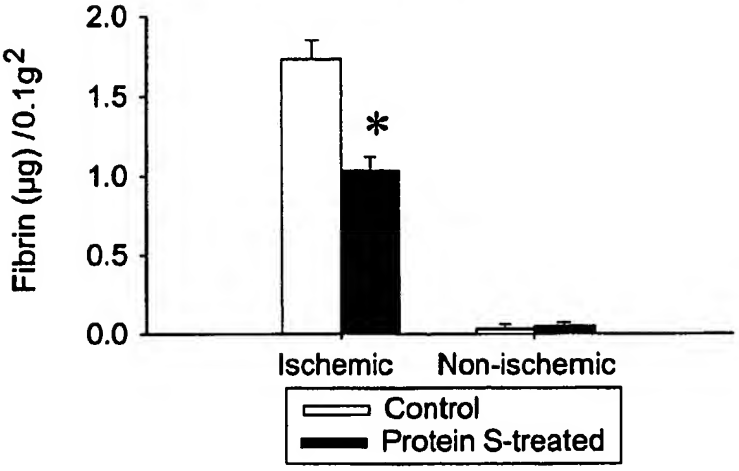
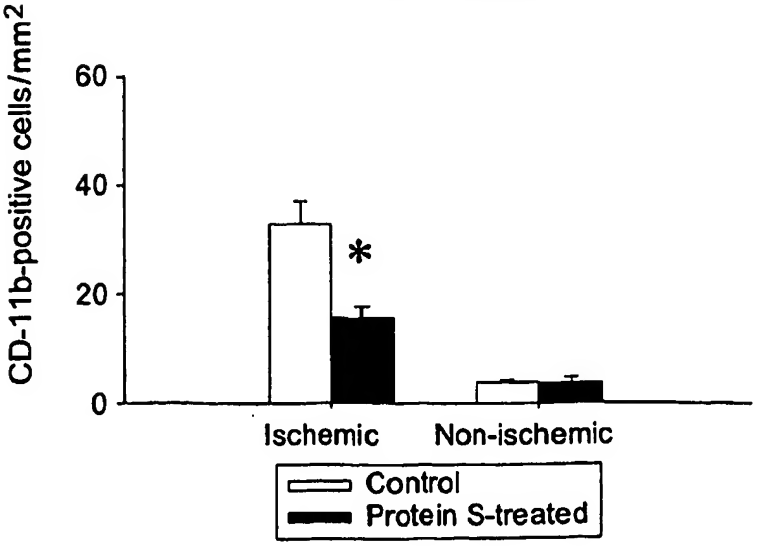


Figure 4C



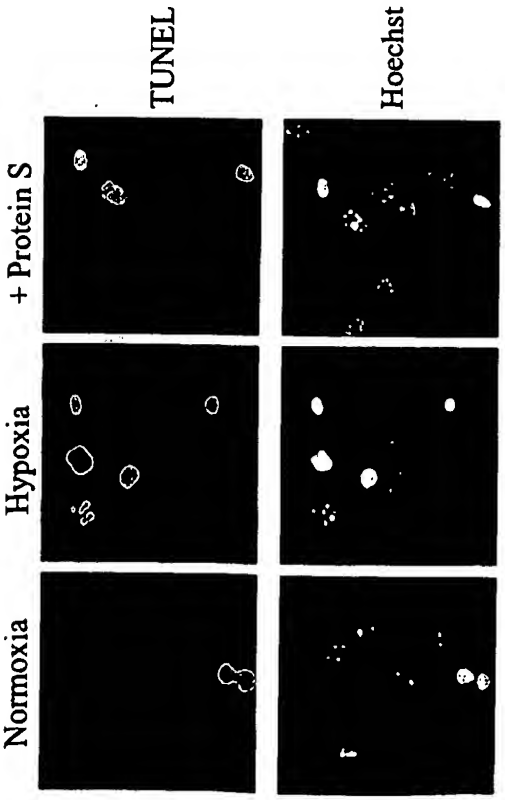


Figure 5A

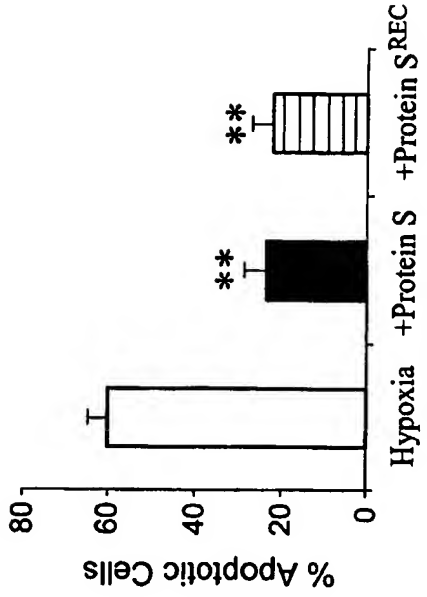


Figure 5B

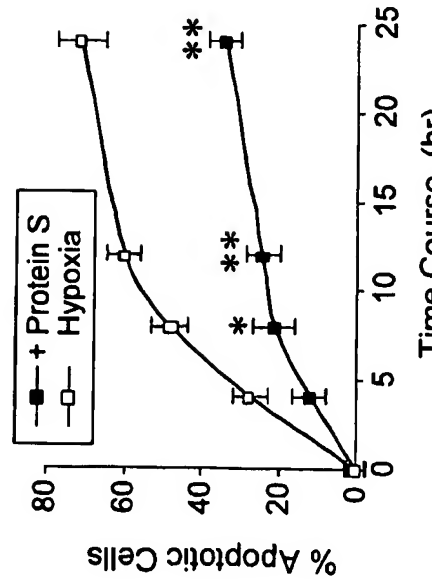


Figure 5C

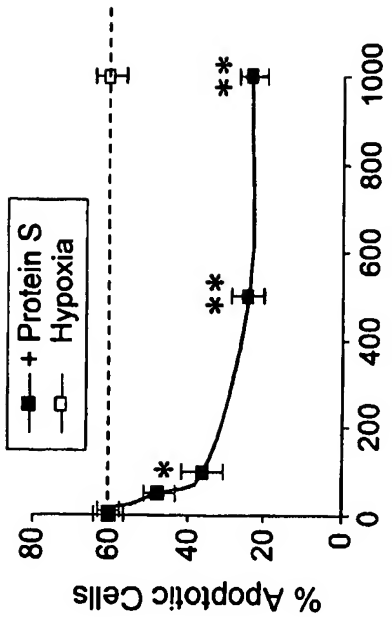


Figure 5D